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(54) Title: USE OF DNA ENCODING PLASTID PYRUVATE DEHYDROGENASE AND BRANCHED CHAIN OXOACID DEHYDROGENASE COMPONENTS TO ENHANCE POLYHYDROXYALKANOATE BIOSYNTHESIS IN PLANTS			
(57) Abstract			
<p>Provided are nucleic acid coding sequences and methods utilizing these sequences for optimizing levels of substrates employed in the biosynthesis of copolymers of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) in plants via manipulation of normal metabolic pathways using recombinant techniques. This optimization is achieved through the use of a variety of wild-type and/or deregulated enzymes involved in the biosynthesis of aspartate family amino acids, and wild-type or deregulated forms of enzymes, such as threonine deaminase, involved in the conversion of threonine to P(3HB-co-3HV) copolymer endproduct. These enzymes are used in conjunction with the E1α, E1β, E2, and E3 subunits of plastid pyruvate dehydrogenase complexes and branched chain oxoacid dehydrogenase complexes or mitochondrial dihydrolipoamide dehydrogenase E3 components to enhance the levels of threonine, 2-oxobuturate (α-keto-butyrate), propionate, propionyl-CoA, β-ketovaleryl-CoA, and β-hydroxyvaleryl-CoA. Also provided are methods for the biological production of P(3HB-co-3HV) copolymer in plants utilizing the enhanced levels of propionyl-CoA produced therein. Introduction into plants of an appropriate β-ketothiolase, a β-ketocetyl-CoA reductase, and a PHA synthase in combinations with the aforementioned enzymes will permit such plants to produce commercially useful amounts of P(3HB-co-3HV) copolymers.</p>			

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Use of DNA Encoding Plastid Pyruvate Dehydrogenase
and Branched Chain Oxoacid Dehydrogenase Components
to Enhance Polyhydroxyalkanoate Biosynthesis in Plants

Background of the Invention

Field of the Invention

The present invention relates to genetically engineered plants. More particularly, the present invention relates to the optimization of substrate pools to facilitate the biosynthetic production of commercially useful polyhydroxyalkanoates (PHAs) in plants.

The present invention especially relates to the production of copolyesters of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV), designated P(3HB-co-3HV) copolymer, and derivatives thereof.

Description of Related Art

Polyhydroxyalkanoates

Polyhydroxyalkanoates are polyesters that accumulate in a wide variety of bacteria. These polymers have properties ranging from stiff and brittle plastics to rubber-like materials, and are biodegradable. Due to these properties, PHAs are an attractive source of non-polluting plastics and elastomers.

Currently, there are approximately a dozen biodegradable plastics in commercial use that possess properties suitable for producing a number of specialty and commodity products (Lindsay, 1992). One such biodegradable plastic in the polyhydroxyalkanoate (PHA) family that is commercially important is Biopol™, a random copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV). This bioplastic is used to produce biodegradable molded material (e.g., bottles), films, coatings, and in drug release applications. Biopol™ is produced via a fermentation process employing the bacterium *Alcaligenes eutrophus* (Byrom, 1987). The

current market price is \$6-7/lb, and the annual production is 1,000 tons. By best estimates, this price is likely to be reduced only about 2-fold via fermentation (Poirier et al., 1995). Competitive synthetic plastics such as polypropylene and polyethylene cost about 35-45¢/lb (Layman, 1994). The annual global demand for polyethylene alone is about 37 million metric tons (Poirier et al., 1995). It is therefore likely that the cost of producing P(3HB-co-3HV) by microbial fermentation will restrict its use to low-volume specialty applications.

Nakamura et al. (1992) reported using threonine (20g/L) as the sole carbon source for the production of P(3HB-co-3HV) copolymer in *A. eutrophus*. These workers initially suggested that the copolymer might form via the degradation of threonine by threonine deaminase, with conversion of the resultant α -ketobutyrate (= 2-oxobutyrate) to propionyl-CoA. However, they ultimately concluded that threonine was utilized directly, without breaking carbon-carbon bonds, to form valeryl-CoA as the 3HV precursor. The nature of this chemical conversion was not described, but since the breaking of carbon-carbon bonds was not postulated to occur, the pathway could not involve threonine deaminase in conjunction with an α -ketoacid decarboxylating step to form propionate or propionyl-CoA. In the experiments of Nakamura et al., the PHA polymer content was very low (< 6% of dry cell weight). This result, in conjunction with the expense of feeding bacteria threonine, makes their approach impractical for the commercial production of P(3HB-co-3HV) copolymer.

Yoon et al. (1995) have shown that growth of *Alcaligenes* sp. SH-69 on a medium supplemented with threonine, isoleucine, or valine resulted in significant increases in the 3HV fraction of the P(3HB-co-3HV) copolymer. In addition to these amino acids, glucose (3% wt/vol) was also added to the growth media. In contrast to the results obtained by Nakamura et al. (1992), growth of *A. eutrophus* under the conditions described by Yoon et al. (1995) did not result in the production of P(3HB-co-3HV) copolymer when the medium was supplemented with threonine as the sole carbon source. From their results, Yoon et al. (1995) implied that the synthetic pathway for the 3HV component in P(3HB-co-3HV) copolymer is likely the same as that described in WO 91/18995 and Steinbüchel and Pieper (1992). This postulated synthetic pathway involves the degradation of isoleucine to propionyl-CoA (Figure 3).

The PHB Biosynthetic Pathway

Polyhydroxybutyrate (PHB) was first discovered in 1926 as a constituent of the bacterium *Bacillus megaterium* (Lemoigne, 1926). Since then, PHAs such as PHB have been found in more than 90 different genera of gram-negative and gram-positive bacteria (Steinbüchel, 1991). These microorganisms produce PHAs using R- β -hydroxyacyl-CoAs as the direct metabolic substrate for a PHA synthase, and produce polymers of R-(3)-hydroxyalkanoates having chain lengths ranging from C3-C14 (Steinbüchel and Valentin, 1995).

To date, the best understood biochemical pathway for PHB production is that found in the bacterium *Alcaligenes eutrophus* (Dawes and Senior, 1973; Slater et al., 1988; Schubert et al., 1988; Peoples and Sinskey, 1989a and 1989b). This pathway, which is also utilized by other microorganisms, is summarized in Figure 1. In this organism, an operon encoding three gene products, i.e.,

PHB synthase, β -ketothiolase, and acetoacetyl-CoA reductase, encoded by the *phbC*, *phbA*, and *phbB* genes, respectively, are required to produce the PHA homopolymer R-polyhydroxybutyrate (PHB).

As further shown in Figure 1, acetyl-CoA is the starting substrate employed in the biosynthetic pathway. This metabolite is naturally available for PHB production in the cytoplasm and plastids of plants.

Poirier et al. (1992) demonstrated that a multi-enzyme pathway can be introduced into plants to produce polyhydroxybutyrate (PHB). In that work, the genes encoding the *Alcaligenes eutrophus* acetoacetyl-CoA reductase (*phbB*) and PHB synthase (*phbC*) genes were introduced into *Arabidopsis thaliana*, where the enzymes were expressed cytoplasmically. A 3-ketothiolase is already expressed in the cytoplasm of *Arabidopsis*. Although PHB was produced in the plants which expressed the three enzymes, the yield was low and the plants were stunted and had reduced seed production.

Nawrath et al. (1994) provided a solution to these problems. There, the genes for the three bacterial PHB enzymes (*phbC*, *phbA*, and *phbB*) were modified to comprise a pea chloroplast targeting peptide ("transit peptide"), which targeted the enzymes to the chloroplast. *Arabidopsis* plants which produced these three enzymes in the chloroplast accumulated large amounts of PHB. There was also no apparent affect of these transgenes, or of the PHB accumulation, on the growth and development of the transgenic plants.

The P(3HB-co-3HV) Copolymer Biosynthetic Pathway

As noted above, P(3HB-co-3HV) random copolymer, commercially known as Biopol™, is produced by fermentation employing *A. eutrophus*. A proposed biosynthetic pathway for P(3HB-co-3HV) copolymer

production is shown in Figure 2. Production of this polymer in plants has been reported (oral presentation by Mitsky et al., 1997).

Since the production of PHB in chloroplasts apparently does not affect plant growth and development as does production of PHB in the cytoplasm (Nawrath et al., 1992), the chloroplast is the preferred site of P(3HB-co-3HV) biosynthesis. The successful production of P(3HB-co-3HV) copolymer in plants thus requires the presence of three PHA biosynthetic enzymes as well as the substrates required for the copolymer biosynthesis (Figure 2), preferably in the plastids. For the 3HB component of the polymer, the substrate naturally exists in chloroplasts in sufficient concentration in the form of acetyl-CoA (Nawrath et al., 1994). However, this is not true for the 3HV component of the polymer, where the starting substrate is propionyl-CoA. Figure 3 is an overview of enzyme pathways which are related to the provision of these substrates. The engineering of plants to generate sufficient chloroplast pools of propionyl-CoA, along with the proper PHA biosynthetic enzymes (i.e., a β -ketothiolase, a β -ketoacyl-CoA reductase, and a PHA synthase), makes it possible to produce copolyesters of poly(3HB-co-3HV) in these organisms.

Methods for optimization of PHB and P(3HB-co-3HV) production in various crop plants are disclosed in Gruys et al. (1998). A major focus in that invention is the optimization of the substrate pools for P(3HB-co-3HV), in order to provide 2-ketobutyrate and propionyl-CoA to the site of copolymer synthesis. Gruys et al. (1998) also discusses exploring the potential use of a pyruvate dehydrogenase complex and a branched chain oxoacid dehydrogenase complex to convert 2-oxobutyrate to propionyl-CoA.

Gruys et al. (1998) also provides methods for the optimization of β -ketothiolase, β -ketoacyl-CoA reductase, and PHA synthase activities in plants and bacteria. It was determined therein that the *A. eutrophus* β -ketothiolase PhbB was metabolically blocked from producing P(3HB-co-3HV) due to its inability to utilize propionyl-CoA with acetyl-CoA to produce 3-ketovaleryl-CoA (see Figure 2). However, Gruys et al. (1998) demonstrated that another *A. eutrophus* β -ketothiolase, designated BktB, is able to produce 3-ketovaleryl-CoA from propionyl-CoA and acetyl-CoA. Therefore, BktB is a preferred β -ketothiolase for the production of P(3HB-co-3HV). Gruys et al. also demonstrated that other β -ketothiolases are able to produce 3-keto-valeryl-CoA from propionyl-CoA and acetyl-CoA. These are: another *A. eutrophus* β -ketothiolase, designated pAE65, and two β -ketothiolases from *Zoogloea ramigera*, designated "A" and "B".

Gruys et al. (1998) noted that the sources of the three copolymer biosynthetic enzymes may encompass a wide range of organisms, including, for example, *Alcaligenes eutrophus*, *Alcaligenes faecalis*, *Aphanothece* sp., *Azotobacter vinelandii*, *Bacillus cereus*, *Bacillus megaterium*, *Beijerinckia indica*, *Derxia gummosa*, *Methylobacterium* sp., *Microcoleus* sp., *Nocardia corallina*, *Pseudomonas cepacia*, *Pseudomonas extorquens*, *Pseudomonas oleovorans*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum* (Brandl et al., 1990; Doi, 1990), and *Thiocapsa pfennigii*.

Pyruvate Dehydrogenase Complex

The pyruvate dehydrogenase complex (PDC) is a large multi-enzyme structure composed of three primary component enzymes, pyruvate dehydrogenase (PDH) (E1, EC 1.2.41); dihydrolipoamide acetyltransferase (E2, EC 2.3.1.12); and dihydrolipoamide dehydrogenase (E3, EC

1.8.1.4) (Reed, 1974). In the well-characterized mammalian complex, 60 subunits of E2 comprise the central core, and the E1 and E3 components decorate the outer surface of this core (Patel et al., 1990). E1 is a heterotetramer composed of two α and two β subunits. The E3 component, a homodimer, associates with the complex via an E3 binding protein (Gopalakrishnan, 1989).

The PDC catalyzes the irreversible oxidative decarboxylation of pyruvate according to the equation:



In mitochondria, this reaction represents the irreversible commitment of carbon to the citric acid cycle, and therefore is a logical point for regulation. Previous experiments have shown that plant mitochondrial PDC activity is, in fact, regulated by product inhibition, metabolites, and reversible phosphorylation (Randall et al., 1977; Randall et al., 1989; Randall et al., 1996; Budde et al, 1991) as is the mammalian complex (Patel et al., 1990).

In prokaryotes, PDC is localized in the cytoplasm, while in eukaryotes it is within the mitochondrial matrix. Plants, however, are unique in that a second form of the complex exists in the plastids (Reid et al., 1975; Reid et al., 1977; Thompson et al, 1977b). Based upon enzymology (Thompson et al., 1977a; Williams et al., 1979; Camp et al., 1988) and immunochemical analyses (Taylor et al., 1992; Camp et al, 1985) it is clear that plastid PDC is distinct from its mitochondrial counterpart. In plants, *de novo* fatty acid biosynthesis occurs exclusively in the plastids (Miernyk et al., 1983; Kang et al., 1994; Zilket et al., 1969; Drennan et al., 1969; Ohlrogge et al., 1979). The plastid form of PDC can provide the fatty acid precursor, acetyl-CoA (Miernyk et al., 1983; Kang et al., 1994; Grof et al., 1995). The

plastid PDC can also catalyze the oxidative decarboxylation of 2-oxobutyrate to produce propionyl Co-A (Camp et al., 1988; Camp and Randall, 1985).

The cDNAs that encode the E1 α and E1 β subunits of plant mitochondrial PDH have been cloned (Grof et al., 1995; Leuthy et al., 1995; Leuthy et al, 1994). Recently, Reith and Munholland (1995) reported the sequence of the entire plastid genome of the red alga *P. purpurea*. Encoded in this genome are open reading frames homologous to PDH α and β subunits.

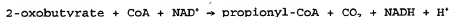
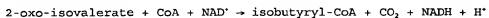
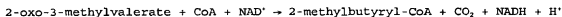
The cDNAs that encode the E2 component of the plant mitochondrial PDC have been similarly cloned (Guan et al., 1995). The sequence of the entire plastid genome of the cyanobacterium *Synechocystis* sp. has also recently been reported (Kaneko et al., 1996).

Branched Chain 2-Oxoacid Dehydrogenase Complex

The branched chain 2-oxoacid dehydrogenase complex (BCOADC) is a highly ordered macromolecular structure composed of three primary component enzymes, a branched chain dehydrogenase or decarboxylase (BCDH or E1; EC 1.2.4.4); dihydrolipoamide transacylase (LTA or E2; no EC number); and dihydrolipoamide dehydrogenase (LipDH or E3; EC 1.8.1.4) (Yeaman, 1989). The mammalian complex is assembled with 24 subunits of E2 as the central cubic core with 4:3:2 symmetry; the E1 and E3 components decorate the outer surface of the E2 core (Yeaman, 1989; Wynn et al., 1996). E1 is a heterotetramer composed of two identical α and two identical β subunits (Pettit et al., 1978). E3 associates loosely with the E2-E1 structure, and is a homodimer (Wynn et al., 1996; Pettit et al., 1978). The mammalian mitochondrial complex is also regulated by a specific E1-kinase and a phospho-E1 phosphatase, which modulate activity by reversible phosphorylation (inactivation) and dephosphorylation

(reactivation). Additional regulation is achieved by product inhibition and modulation of gene expression (Yeaman, 1989; Wynn et al., 1996).

BCOADC catalyzes the irreversible oxidative decarboxylation of the branched-chain 2-oxoacids derived from valine, leucine and isoleucine, as well as 2-oxobutyrate and 4-methyl-2-oxobutyrate, with comparable rates and similar Km values (Yeaman 1989; Wynn et al., 1996; Paxton et al., 1986; Gerbling et al., 1988). The reactions are:



In mammals, BCOADC is found in the mitochondria and is involved in the catabolism of the branched-chain amino acids. The only reports describing BCOADC activity in plants have localized BCOADC to peroxisomes (Gerbling et al., 1988; Gerbling et al., 1989). The proposed function of a peroxisomal BCOADC is to catabolize the branched-chain amino acids during germination and growth, yielding an acyl-CoA product that would be further metabolized by the beta-oxidation pathway localized in the peroxisome (Gerbling et al., 1988; Gerbling et al., 1989).

To provide substrate pools to permit biosynthesis of P(3HB-co-3HV) copolymer in the plastid, there is a need for methods to engineer plants to produce plastid enzymes which convert 2-oxobutyrate to propionyl-CoA.

Summary of the Invention

Accordingly, the present invention provides nucleotide sequences that encode the E1 α and E1 β subunits, and the E2 component of the plastid pyruvate

dehydrogenase complex, as well as the E1 α and E1 β subunits, and the E2 component of the branched chain oxoacid dehydrogenase complex, of *Arabidopsis thaliana*. Methods of utilizing these nucleotide sequences to provide enzymatic activity to convert 2-oxo-butyrate to propionyl-CoA, and to produce P(3HB-co-3HV) copolymer in plants, are also provided.

Accordingly, in a first aspect, the present invention provides an isolated DNA molecule, comprising a nucleotide sequence selected from: (a) the nucleotide sequence shown in SEQ ID NO:1, or the complement thereof; (b) a nucleotide sequence that hybridizes to the nucleotide sequence of (a) under a wash stringency equivalent to 0.5X SSC to 2X SSC, 0.1% SDS, at 55-65°C, and which encodes a polypeptide having enzymatic activity similar to that of *Arabidopsis thaliana* plastid pyruvate dehydrogenase complex E1 α subunit; (c) a nucleotide sequence encoding the same genetic information as the nucleotide sequence of (a), but which is degenerate in accordance with the degeneracy of the genetic code; and (d) a nucleotide sequence encoding the same genetic information as the nucleotide sequence of (b), but which is degenerate in accordance with the degeneracy of the genetic code. Recombinant vectors comprising such isolated DNA molecules, host cells transformed with these vectors, and an isolated polypeptide having the amino acid sequence of SEQ ID NO:2 are also provided.

In another aspect, the present invention provides an isolated DNA molecule, comprising a nucleotide sequence selected from: (a) the nucleotide sequence shown in SEQ ID NO:3, or the complement thereof; (b) a nucleotide sequence that hybridizes to the nucleotide sequence of (a) under a wash stringency equivalent to 0.5X SSC to 2X SSC, 0.1% SDS, at 55-65°C, and which encodes a polypeptide having enzymatic activity similar to that of *Arabidopsis thaliana* plastid pyruvate dehydrogenase

complex E1 β subunit; (c) a nucleotide sequence encoding the same genetic information as the nucleotide sequence of (a), but which is degenerate in accordance with the degeneracy of the genetic code; and (d) a nucleotide sequence encoding the same genetic information as the nucleotide sequence of (b), but which is degenerate in accordance with the degeneracy of the genetic code. Recombinant vectors comprising such isolated DNA molecules, host cells transformed with these vectors, and an isolated polypeptide having the amino acid sequence of SEQ ID NO.:4 are also provided.

In another aspect, the present invention provides an isolated DNA molecule, comprising a nucleotide sequence selected from: (a) the nucleotide sequence shown in SEQ ID NO.:5, or the complement thereof; (b) a nucleotide sequence that hybridizes to the nucleotide sequence of (a) under a wash stringency equivalent to 0.5X SSC to 2X SSC, 0.1% SDS, at 55-65°C, and which encodes a polypeptide having enzymatic activity similar to that of *Arabidopsis thaliana* plastid pyruvate dehydrogenase complex E2 component; (c) a nucleotide sequence encoding the same genetic information as the nucleotide sequence of (a), but which is degenerate in accordance with the degeneracy of the genetic code; and (d) a nucleotide sequence encoding the same genetic information as the nucleotide sequence of (b), but which is degenerate in accordance with the degeneracy of the genetic code. Recombinant vectors comprising such isolated DNA molecules, host cells transformed with these vectors, and an isolated polypeptide having the amino acid sequence of SEQ ID NO.:6 are also provided.

In a further aspect, the present invention provides an isolated DNA molecule, comprising a nucleotide sequence selected from: (a) the nucleotide sequence shown in SEQ ID NO.:11, or the complement thereof; (b) a nucleotide sequence that hybridizes to the nucleotide

sequence of (a) under a wash stringency equivalent to 0.5X SSC to 2X SSC, 0.1% SDS, at 55-65°C, and which encodes a polypeptide having enzymatic activity similar to that of *Arabidopsis thaliana* branched chain 2-oxoacid dehydrogenase complex E1 α subunit; (c) a nucleotide sequence encoding the same genetic information as the nucleotide sequence of (a), but which is degenerate in accordance with the degeneracy of the genetic code; and (d) a nucleotide sequence encoding the same genetic information as the nucleotide sequence of (b), but which is degenerate in accordance with the degeneracy of the genetic code. Recombinant vectors comprising such isolated DNA molecules, host cells transformed with these vectors, and an isolated polypeptide having the amino acid sequence of SEQ ID NO.:12 are also provided.

In another aspect, the present invention provides an isolated DNA molecule, comprising a nucleotide sequence selected from: (a) the nucleotide sequence shown in SEQ ID NO.:13, or the complement thereof; (b) a nucleotide sequence that hybridizes to the nucleotide sequence of (a) under a wash stringency equivalent to 0.5X SSC to 2X SSC, 0.1% SDS, at 55-65°C, and which encodes a polypeptide having enzymatic activity similar to that of *Arabidopsis thaliana* branched chain 2-oxoacid dehydrogenase complex E1 β subunit; (c) a nucleotide sequence encoding the same genetic information as the nucleotide sequence of (a), but which is degenerate in accordance with the degeneracy of the genetic code; and (d) a nucleotide sequence encoding the same genetic information as the nucleotide sequence of (b), but which is degenerate in accordance with the degeneracy of the genetic code. Recombinant vectors comprising such isolated DNA molecules, host cells transformed with these vectors, and an isolated polypeptide having the amino acid sequence of SEQ ID NO.:14 are also provided.

In another aspect, the present invention provides the foregoing isolated DNA molecules encoding a polypeptide having enzymatic activity similar to that of *Arabidopsis thaliana* branched chain 2-oxoacid dehydrogenase complex E1 β subunit, but in which the naturally occurring branched chain oxoacid dehydrogenase complex E2 component binding region thereof is replaced with the E2 component binding region of a plastid pyruvate dehydrogenase complex E1 β subunit. The plastid pyruvate dehydrogenase complex E1 β subunit can have the sequence shown in SEQ ID NO.:3. Recombinant vectors comprising such isolated DNA molecules, host cells transformed with these vectors, and the isolated polypeptide are also provided.

In yet another aspect, the present invention provides an isolated DNA molecule, comprising a nucleotide sequence selected from: (a) the nucleotide sequence shown in SEQ ID NO:15, or the complement thereof; (b) a nucleotide sequence that hybridizes to the nucleotide sequence of (a) under a wash stringency equivalent to 0.5X SSC to 2X SSC, 0.1% SDS, at 55-65°C, and which encodes a polypeptide having enzymatic activity similar to that of *Arabidopsis thaliana* branched chain 2-oxoacid dehydrogenase complex E2 component; (c) a nucleotide sequence encoding the same genetic information as the nucleotide sequence of (a), but which is degenerate in accordance with the degeneracy of the genetic code; and (d) a nucleotide sequence encoding the same genetic information as the nucleotide sequence of (b), but which is degenerate in accordance with the degeneracy of the genetic code. Recombinant vectors comprising such isolated DNA molecules, host cells transformed with these vectors, and an isolated polypeptide having the amino acid sequence of SEQ ID NO.:16 are also provided.

In another aspect, the present invention provides a plant, a plastid of which comprises the following polypeptides: an enzyme that enhances the biosynthesis of 2-oxobutyrate; a branched chain oxoacid dehydrogenase complex E1 α subunit; a branched chain oxoacid dehydrogenase complex E1 β subunit; and a branched chain oxoacid dehydrogenase complex E2 component. The branched chain oxoacid dehydrogenase complex E1 α subunit can have the sequence shown in SEQ ID NO.:12, the branched chain oxoacid dehydrogenase complex E1 β subunit can have the sequence shown in SEQ ID NO.:14, or the branched chain oxoacid dehydrogenase complex E2 component can have the sequence shown in SEQ ID NO.:16. In such plant, the plastid can further comprise the following polypeptides: a β -keto-thiolase; a β -ketoacyl-CoA reductase; and a polyhydroxy-alkanoate synthase. The genome of such plant can comprise introduced DNAs encoding these polypeptides, wherein each of the introduced DNAs is operatively linked to a targeting peptide coding region capable of directing transport of the polypeptide encoded thereby into a plastid. A method of producing P(3HB-co-3HV) copolymer comprises growing such plant, and recovering P(3HB-co-3HV) copolymer produced thereby.

In another aspect, the present invention comprises a plant, a plastid of which comprises the following polypeptides: an enzyme that enhances the biosynthesis of 2-oxobutyrate; a branched chain oxoacid dehydrogenase complex E1 α subunit; a branched chain oxoacid dehydrogenase complex E1 β subunit; a branched chain oxoacid dehydrogenase complex E2 component; and a dihydrolipoamide dehydrogenase E3 component, which can be mitochondrially-derived. In such plant, the branched chain oxoacid dehydrogenase complex E1 α subunit can have the sequence shown in SEQ ID NO.:12, the branched chain oxoacid dehydrogenase complex E1 β subunit can have the sequence shown in SEQ ID NO.:14, or the branched chain

oxoacid dehydrogenase complex E2 component can have the sequence shown in SEQ ID NO.:16. In such plant, the plastid can further comprise the following polypeptides: a β -keto-thiolase; a β -ketoacyl-CoA reductase; and a polyhydroxy-alkanoate synthase. The genome of such plant can comprise introduced DNAs encoding these polypeptides, wherein each of the introduced DNAs is operatively linked to a targeting peptide coding region capable of directing transport of the polypeptide encoded thereby into a plastid. A method of producing P(3HB-co-3HV) copolymer comprises growing such plant, and recovering P(3HB-co-3HV) copolymer produced thereby.

In yet another aspect, the present invention provides a plant, a plastid of which comprises the following polypeptides: an enzyme that enhances the biosynthesis of 2-oxobutyrate; a branched chain oxoacid dehydrogenase complex E1 α subunit; and a branched chain oxoacid dehydrogenase complex E1 β subunit, the naturally occurring E2 binding region of which is replaced with the E2 binding region of a plastid pyruvate dehydrogenase complex E1 β subunit. In such plant, the branched chain oxoacid dehydrogenase complex E1 α subunit can have the sequence shown in SEQ ID NO.:12. Furthermore, in such plant, the plastid can further comprise the following polypeptides: a β -ketothiolase; a β -ketoacyl-CoA reductase; and a poly-hydroxyalkanoate synthase. In such plant, the genome can comprise introduced DNAs encoding these polypeptides, wherein each of the introduced DNAs is operatively linked to a targeting peptide coding region capable of directing transport of the polypeptide encoded thereby into a plastid. A method of producing P(3HB-co-3HV) copolymer comprises growing such plant, and recovering P(3HB-co-3HV) copolymer produced thereby.

Further scope of the applicability of the present invention will become apparent from the detailed description and drawings provided below. However, it

should be understood that the following detailed description and examples, while indicating preferred embodiments of the invention, are given by way of illustration only since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

The above and other objects, features, and advantages of the present invention will be better understood from the following detailed description taken in conjunction with the accompanying drawings, all of which are given by way of illustration only, and are not limitative of the present invention, in which:

Figure 1 shows the biochemical steps involved in the production of PHB from acetyl-CoA catalyzed by the *A. eutrophus* PHB biosynthetic enzymes.

Figure 2 shows the biochemical steps involved in the production of P(3HB-co-3HV) copolymer from acetyl-CoA and propionyl-CoA catalyzed by PHA biosynthetic enzymes of *A. eutrophus*.

Figure 3 summarizes the pathways discussed herein that are involved in the production of P(3HB-co-3HV) copolymer, including enzymes that can be used to enhance 2-oxobutyrate biosynthesis.

Figure 4 shows Southern analyses of genomic DNA isolated from mature *A. thaliana* leaves. Each lane was loaded with 10 μ g of DNA digested with *Bam*HI, *Hind* III, *Sal* I, *Eco* RI or *Xba* I as indicated. Fig. 5A and 5B, genomic Southern blots hybridized with random primed probes generated from gel-excised *El* α and *El* β cDNAs respectively. (α^{32} P)-dCTP was incorporated using an oligolabelling kit (Pharmacia, Uppsala, Sweden). The positions of λ DNA markers digested with *Hind* III are indicated to the left of the figure.

Figure 5 shows Northern blot analyses of *A. thaliana* RNA. Total RNA was isolated from young leaves of *A. thaliana* plants. 10 μ g of total RNA was run on formaldehyde gels then transferred to nylon membranes. Probes were prepared as described in the legend for Figure 5. RNA markers were used to determine the sizes of the hybridizing bands.

Figures 6A and 6B show dendrogram analyses of the deduced amino acid sequence of PDH E1 α and E1 β subunits, respectively. Abbreviations and accession numbers to the sequences are: *P. p.*, *Porphyra purpurea* odp (U38804); *S. sp.*, *Synechocystis sp.* (D90915); *A. t.*, *Arabidopsis thaliana* (U21214, U09137); *P. s.*, *Pisum sativum* (U51918, U56697); *H. s.*, *Homo sapiens* (L13318, D90086); *R. r.*, *Rattus rattus* (Z12158, P49432); *S. c.*, *Saccharomyces cerevisiae* (P16387, M98476); *A. s.*, *Ascaris suum* (M76554, M38017); *M. gen.*, *Mycoplasma genitalium* (U39706); *M. c.*, *Mycoplasma capricolum* (U62057); *B. su.*, *Bacillus subtilis* (M57435); and *B. s.*, *Bacillus stearothermophilus* (X53560). Dendrogram analyses was accomplished with GeneWorks CLUSTAL V method (IntelliGenetics, Mountain View, CA). CLUSTAL V parameters were as follows: cost to open gap = 5, cost to lengthen gap = 25, gap penalty = 3, number of top diagonals = 5, window size = 5, PAM matrix = PAM250, K-tuple = 1, and consensus cutoff = 50%.

Figures 7A-7E shows schematics (Constructs 1-5) for engineering the BCOADC subunits to be targeted to the plastid and to form a hybrid complex, as described in Examples 6 and 7.

Detailed Description of the Invention

The following detailed description is provided to aid those skilled in the art in practicing the present invention. Even so, this detailed description should not be construed to unduly limit the present invention as modifications and variations in the embodiments discussed

herein can be made by those of ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

The contents of each of the references cited herein, including those of the references cited within these primary references, are herein incorporated by reference in their entirety.

The production of P(3HB-co-3HV) in plants requires the substrates propionyl-CoA and acetyl-CoA, and three enzymes which convert these substrates to P(3HB-co-3HV): a β -ketothiolase, a β -ketoacyl-CoA reductase, and a PHA synthase. β -ketothiolase is normally present in the plant cytoplasm, but not in the plastids. Acetyl-CoA is normally present in the cytoplasm and the plastids. All of the other required components must be introduced into the plant, preferably into the plastids.

Gruys et al. (1998) discusses several ways in which 2-oxobutyrate can be provided in the plant. One way is through the manipulation of various wild-type and/or deregulated enzymes involved in the biosynthesis of aspartate family amino acids in order to increase threonine levels, thereby creating a larger substrate pool for threonine deaminase to convert to 2-oxobutyrate (Figure 3), and wild-type or deregulated forms of enzymes, such as threonine deaminase, involved in the conversion of threonine to P(3HB-co-3HV) copolymer endproduct. Enzymes which can be manipulated to enhance the threonine pool include aspartate kinase, homoserine dehydrogenase, and threonine synthase. The threonine substrate pool can be enhanced by overexpression of these enzymes, or by the use of deregulated forms of these enzymes, such as lysine-deregulated aspartate kinase.

Threonine deaminase, which converts threonine to 2-oxobutyrate, is another enzyme which can be utilized in the production of 2-oxobutyrate. Deregulated mutants and

natural deregulated forms of threonine deaminase can be used to increase 2-oxobutyrate pools at the site of copolymer biosynthesis.

Gruys et al. (1998), at Example 6, also discuss several ways in which the PDC and/or the BCOADC, or their substrate pools, can be manipulated to provide effective conversion of 2-oxobutyrate to propionyl-CoA. The native plastid PDC is able to perform this conversion at a low level. However, this complex can provide levels of propionyl-CoA sufficient for P(3HB-co-3HV) if the levels of 2-oxobutyrate are sufficient, or if portions of the BCOADC are employed to form a hybrid complex. The plastid PDC might also be genetically manipulated to be more effective in providing propionyl-CoA (Gruys et al., 1998).

The present invention provides nucleotide sequences that encode the E1 α and E1 β subunits, and the E2 component, of the plastid pyruvate dehydrogenase complex, and the E1 α and E1 β subunits, and the E2 component, of the branched chain oxoacid dehydrogenase complex of *Arabidopsis thaliana*. These nucleotide sequences and the enzymatic polypeptides encoded thereby can be introduced into plants in various combinations with coding sequences for the foregoing enzymes in order to enhance the conversion of threonine to 2-oxobutyrate, propionate, propionyl-CoA, β -ketovaleryl-CoA, and β -hydroxyvaleryl-CoA. Introduction into such plants of nucleic acid sequences encoding an appropriate β -keto-thiolase, a β -ketoacyl-CoA reductase, and a PHA synthase will permit such transgenic plants to utilize the increased β -hydroxyvaleryl-CoA substrate in the production of P(3HB-co-3HV) copolymer.

Definitions

The following definitions are provided to aid those skilled in the art in understanding the detailed description of the present invention.

" β -ketoacyl-CoA reductase" refers to a β -ketoacyl-CoA reducing enzyme that can convert a β -ketoacyl-CoA substrate to its corresponding β -hydroxyacyl-CoA product using, for example, NADH or NADPH as the reducing cosubstrate. An example is the PhbB acetoacetyl-CoA reductase of *A. eutrophus*.

" β -ketothiolase" refers to an enzyme that catalyzes the thiolytic cleavage of a β -ketoacyl-CoA, requiring free CoA, to form two acyl-CoA molecules. However, the term β -ketothiolase as used herein also refers to enzymes that catalyze the condensation of two acyl-CoA molecules to form β -ketoacyl-CoA and free CoA, i.e., the reverse of the thiolytic cleavage reaction.

"CoA" refers to coenzyme A.

"C-terminal" refers to the region of a peptide, polypeptide, or protein chain from the middle thereof to the end that carries the amino acid having a free α carboxyl group.

"Deregulated enzyme" refers to an enzyme that has been modified, for example by mutagenesis, wherein the extent of feedback inhibition of the catalytic activity of the enzyme by a metabolite is reduced such that the enzyme exhibits enhanced activity in the presence of said metabolite compared to the unmodified enzyme. Some organisms possess deregulated forms of such enzymes as the naturally occurring, wild-type form.

The term "DNA encoding" or "encoding DNA" refers to chromosomal DNA, plasmid DNA, cDNA, plastid DNA, or synthetic DNA which codes for expression for any of the enzymes discussed herein.

The term "genome" as it applies to bacteria encompasses both the chromosome and plasmids within a bacterial host cell. Unless specified, the term "genome" as it applies to plant cells encompasses not only chromosomal or nuclear DNA found within the nucleus, but organellar DNA found within subcellular components of the cell. DNAs of the present invention introduced into plant cells can therefore be either chromosomally-integrated or organelle-localized, unless specified (e.g. "plastid genome").

The term "mutein" refers to a mutant form of a peptide, polypeptide, or protein.

"N-terminal" refers to the region of a peptide, polypeptide, or protein chain from the amino acid having a free α -amino group to the middle of the chain.

"Operably linked" refers to two amino acid or nucleotide sequences wherein one of the sequences operates to affect a characteristic of the other sequence. In the case of nucleotide sequences, for example, a promoter "operably linked" to a structural coding sequence acts to drive expression of the latter.

"Overexpression" refers to the expression of a polypeptide or protein encoded by a DNA introduced into a host cell, wherein said polypeptide or protein is either not normally present in the host cell, or wherein said polypeptide or protein is present in said host cell at a higher level than that normally expressed from the endogenous gene encoding said polypeptide or protein.

The term "plastid" refers to the class of plant cell organelles that includes amyloplasts, chloroplasts, chromoplasts, elaioplasts, eoplasts, etioplasts, leucoplasts, and proplastids. These organelles are self-replicating, and contain what is commonly referred to as the chloroplast genome, a circular DNA molecule

that ranges in size from about 120 to about 217 kb, depending upon the plant species, and which usually contains an inverted repeat region (Fosket, 1994).

The term "polyhydroxyalkanoate (PHA) synthase" refers to enzymes that convert β -hydroxyacyl-CoAs to polyhydroxyalkanoates and free CoA.

"Targeting sequence" refers to a nucleotide sequence which, when expressed (forming a "targeting peptide"), directs the export of an attached polypeptide to a particular cellular location, such as the chloroplast (e.g. "chloroplast targeting sequence"). The words "signal" or "transit" are equivalent to "targeting" in this context.

Production of Transgenic Plants Capable of Producing P(3HB-co-3HV) Copolymer

PHA synthesis in plants can be optimized in accordance with the present invention by expressing DNAs encoding β -ketothiolase, β -acyl-CoA reductase, and PHA synthase in conjunction with various portions and combinations of precursor-producing enzymes, including the sequences encoding portions of the plastid PDC and the BCOADC provided herein, as discussed in the Examples below.

Plant Vectors

In plants, transformation vectors capable of introducing encoding DNAs involved in PHA biosynthesis are easily designed, and generally contain one or more DNA coding sequences of interest under the transcriptional control of 5' and 3' regulatory sequences. Such vectors generally comprise, operatively linked in sequence in the 5' to 3' direction, a promoter sequence that directs the transcription of a downstream heterologous structural DNA in a plant; optionally, a 5' non-translated leader sequence; a nucleotide sequence

that encodes a protein of interest; and a 3' non-translated region that encodes a polyadenylation signal which functions in plant cells to cause the termination of transcription and the addition of polyadenylate nucleotides to the 3' end of the mRNA encoding said protein. Plant transformation vectors also generally contain a selectable marker. Typical 5'-3' regulatory sequences include a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal. Vectors for plant transformation have been reviewed in Rodriguez et al. (1988), Glick et al. (1993), and Croy (1993).

Plant Promoters

Plant promoter sequences can be constitutive or inducible, environmentally- or developmentally-regulated, or cell- or tissue-specific. Often-used constitutive promoters include the CaMV 35S promoter (Odell et al., 1985), the enhanced CaMV 35S promoter, the Figwort Mosaic Virus (FMV) promoter (Richins et al., 1987), the mannopine synthase (*mas*) promoter, the nopaline synthase (*nos*) promoter, and the octopine synthase (*ocs*) promoter. Useful inducible promoters include heat-shock promoters (Ou-Lee et al., 1986; Ainley et al., 1990), a nitrate-inducible promoter derived from the spinach nitrite reductase gene (Back et al., 1991), hormone-inducible promoters (Yamaguchi-Shinozaki et al., 1990; Kares et al., 1990), and light-inducible promoters associated with the small subunit of RuBP carboxylase and LHCP gene families (Kuhlemeier et al., 1989; Feinbaum et al., 1991; Weisshaar et al., 1991; Lam and Chua, 1990; Castresana et al., 1988; Schulze-Lefert et al., 1989). Examples of useful tissue-specific, developmentally-regulated promoters include the β -conglycinin 7S promoter (Doyle et al., 1986; Slighon

and Beachy, 1987), and seed-specific promoters (Knutzon et al., 1992; Bustos et al., 1991; Lam and Chua, 1991; Stayton et al., 1991). Plant functional promoters useful for preferential expression in seed plastids include those from plant storage protein genes and from genes involved in fatty acid biosynthesis in oilseeds. Examples of such promoters include the 5' regulatory regions from such genes as napin (Kridl et al., 1991), phaseolin, zein, soybean trypsin inhibitor, ACP, stearyl-ACP desaturase, and oleosin. Seed-specific gene regulation is discussed in EP 0 255 378. Promoter hybrids can also be constructed to enhance transcriptional activity (Hoffman, U.S. Patent No. 5,106,739), or to combine desired transcriptional activity and tissue specificity.

A factor to be considered in the choice of promoters is the timing of availability of the necessary substrates during expression of the PHA biosynthetic enzymes. For example, if P(3HB-co-3HV) copolymer is produced in seeds from threonine, the timing of threonine biosynthesis and the amount of free threonine are important considerations. Karchi et al. (1994) have reported that threonine biosynthesis occurs rather late in seed development, similar to the timing of seed storage protein accumulation. For example, if enzymes involved in P(3HB-co-3HV) copolymer biosynthesis are expressed from the 7S seed-specific promoter, the timing of expression thereof will be concurrent with threonine accumulation.

Plant Transformation and Regeneration

A variety of different methods can be employed to introduce such vectors into plant protoplasts, cells, callus tissue, leaf discs, meristems, etc., to generate transgenic plants, including *Agrobacterium*-mediated transformation, particle gun delivery, microinjection,

electroporation, polyethylene glycol-mediated protoplast transformation, liposome-mediated transformation, etc. (reviewed in Potrykus, 1991). In general, transgenic plants comprising cells containing and expressing DNAs encoding enzymes facilitating PHA biosynthesis can be produced by transforming plant cells with a DNA construct as described above via any of the foregoing methods; selecting plant cells that have been transformed on a selective medium; regenerating plant cells that have been transformed to produce differentiated plants; and selecting a transformed plant which expresses the enzyme-encoding nucleotide sequence.

Constitutive overexpression of, for example, a deregulated threonine deaminase employing the CaMV 35S or FMV promoter might potentially starve plants of certain amino acids, especially those of the aspartate family. If such starvation occurs, the negative effects may be avoided by supplementing the growth and cultivation media employed in the transformation and regeneration procedures with appropriate amino acids. By supplementing the transformation/regeneration media with aspartate family amino acids (aspartate, threonine, lysine, and methionine), the uptake of these amino acids into the plant can reduce any potential starvation effect caused by an overexpressed threonine deaminase. Supplementation of the media with such amino acids might thereby prevent any negative selection, and therefore any adverse effect on transformation frequency, due to the overexpression of a deregulated threonine deaminase in the transformed plant.

The encoding DNAs can be introduced either in a single transformation event (all necessary DNAs present on the same vector), a co-transformation event (all necessary DNAs present on separate vectors that are introduced into plants or plant cells simultaneously), or by independent transformation events (all necessary DNAs

present on separate vectors that are introduced into plants or plant cells independently). Traditional breeding methods can subsequently be used to incorporate the entire pathway into a single plant. Successful production of the PHA polyhydroxybutyrate in cells of *Arabidopsis* has been demonstrated by Poirier et al. (1992), and in plastids thereof by Nawrath et al. (1994).

Specific methods for transforming a wide variety of dicots and obtaining transgenic plants are well documented in the literature (Gasser and Fraley, 1989; Fisk and Dandekar, 1993; Christou, 1994; and the references cited therein).

Successful transformation and plant regeneration have been achieved in the monocots as follows: asparagus (*Asparagus officinalis*; Bytebier et al. 1987); barley (*Hordeum vulgare*; Wan and Lemaux 1994); maize (*Zea mays*; Rhodes et al., 1988; Gordon-Kamm et al., 1990; Fromm et al., 1990; Koziel et al., 1993); oats (*Avena sativa*; Somers et al., 1992); orchardgrass (*Dactylis glomerata*; Horn et al., 1988); rice (*Oryza sativa*, including indica and japonica varieties; Toriyama et al., 1988; Zhang et al., 1988; Luo and Wu 1988; Zhang and Wu 1988; Christou et al., 1991); rye (*Secale cereale*; De la Pena et al., 1987); sorghum (*Sorghum bicolor*; Cassas et al. 1993); sugar cane (*Saccharum* spp.; Bower and Birch 1992); tall fescue (*Festuca arundinacea*; Wang et al. 1992); turfgrass (*Agrostis palustris*; Zhong et al., 1993); and wheat (*Triticum aestivum*; Vasil et al. 1992; Weeks et al. 1993; Becker et al. 1994).

Host Plants

Particularly useful plants for PHA copolymer production include those that produce carbon substrates which can be employed for PHA biosynthesis, including tobacco, wheat, potato, *Arabidopsis*, and high oil seed plants such as corn, soybean, canola, oil seed rape,

sunflower, flax, and peanut. Polymers that can be produced in this manner include copolymers incorporating both short chain length and medium chain length monomers, such as P(3HB-co-3HV) copolymer.

If the host plant of choice does not produce the requisite fatty acid substrates in sufficient quantities, it can be modified, for example by mutagenesis or genetic transformation, to block or modulate the glycerol ester and fatty acid biosynthesis or degradation pathways so that it accumulates the appropriate substrates for PHA production.

Plastid Targeting of Expressed Enzymes for PHA Biosynthesis

PHA polymer can be produced in plants either by expression of the appropriate enzymes in the cytoplasm (Poirier et al., 1992) by the methods described above, or more preferably, in plastids, where higher levels of PHA production can be achieved (Nawrath et al., 1994). As demonstrated by the latter group, targeting of β -ketothiolase, acetoacetyl-CoA reductase, and PHB synthase to plastids of *Arabidopsis thaliana* results in the accumulation of high levels of PHB in the plastids without any readily apparent deleterious effects on plant growth and seed production. As branched-chain amino acid biosynthesis occurs in plant plastids (Bryan, 1980; Galili, 1995), overexpression therein of plastid-targeted enzymes, including a deregulated form of threonine deaminase, is expected to facilitate the production of elevated levels of 2-oxobutyrate and propionyl-CoA. The latter can be condensed with acetyl-CoA by β -ketothiolase to form 3-ketovaleryl-CoA, which can then be further metabolized by a β -keto-acyl-CoA reductase to 3-hydroxyvaleryl-CoA, the precursor of the C5 subunit of P(3HB-co-3HV) copolymer. As there is a

high carbon flux through acetyl-CoA in plastids, especially in seeds of oil-accumulating plants such as oilseed rape (*Brassica napus*), canola (*Brassica rapa*, *Brassica campestris*, *Brassica carinata*, and *Brassica juncea*), soybean (*Glycine max*), flax (*Linum usitatissimum*), and sunflower (*Helianthus annuus*) for example, targeting of the gene products of desired encoding DNAs to leucoplasts of seeds, or transformation of seed leucoplasts and expression therein of these encoding DNAs, are attractive strategies for achieving high levels of PHA biosynthesis in plants.

All of the enzymes discussed herein can be modified for plastid targeting by employing plant cell nuclear transformation constructs wherein DNA coding sequences of interest are fused to any of the available transit peptide sequences capable of facilitating transport of the encoded enzymes into plant plastids (partially summarized in von Heijne et al., 1991), and driving expression by employing an appropriate promoter. The sequences that encode a transit peptide region can be obtained, for example, from plant nuclear-encoded plastid proteins, such as the small subunit (SSU) of ribulose biphosphate carboxylase, plant fatty acid biosynthesis related genes including acyl carrier protein (ACP), stearyl-ACP desaturase, β -ketoacyl-ACP synthase and acyl-ACP thioesterase, or LHCPII genes. The encoding sequence for a transit peptide effective in transport to plastids can include all or a portion of the encoding sequence for a particular transit peptide, and may also contain portions of the mature protein encoding sequence associated with a particular transit peptide. Numerous examples of transit peptides that can be used to

deliver target proteins into plastids exist, and the particular transit peptide encoding sequences useful in the present invention are not critical as long as delivery into a plastid is obtained. Proteolytic processing within the plastid then produces the mature enzyme. This technique has proven successful not only with enzymes involved in PHA synthesis (Nawrath et al., 1994), but also with neomycin phosphotransferase II (NPT-II) and CP4 EPSPS (Padgett et al., 1995), for example.

Of particular interest are transit peptide sequences derived from enzymes known to be imported into the leucoplasts of seeds. Examples of enzymes containing useful transit peptides include those related to lipid biosynthesis (e.g., subunits of the plastid-targeted dicot acetyl-CoA carboxylase, biotin carboxylase, biotin carboxyl carrier protein, α -carboxytransferase, plastid-targeted monocot multifunctional acetyl-CoA carboxylase (Mr, 220,000); plastidic subunits of the fatty acid synthase complex (e.g., acyl carrier protein (ACP), malonyl-ACP synthase, KASI, KASII, KASIII, etc.); steroyl-ACP desaturase; thioesterases (specific for short, medium, and long chain acyl ACP); plastid-targeted acyl transferases (e.g., glycerol-3-phosphate: acyl transferase); enzymes involved in the biosynthesis of aspartate family amino acids; phytoene synthase; gibberellic acid biosynthesis (e.g., *ent*-kaurene synthases 1 and 2); sterol biosynthesis (e.g., hydroxy methyl glutaryl-coA reductase); and carotenoid biosynthesis (e.g., lycopene synthase).

Exact translational fusions to the transit peptide of interest may not be optimal for protein import into the plastid. By creating translational fusions of any

of the enzymes discussed herein to the precursor form of a naturally imported protein or C-terminal deletions thereof, one would expect that such translational fusions would aid in the uptake of the engineered precursor protein into the plastid. For example, Nawrath et al., (1994) used a similar approach to create the vectors employed to introduce the PHB biosynthesis genes of *A. eutrophus* into *Arabidopsis*.

It is therefore fully expected that targeting of the enzymes discussed herein to leaf chloroplasts or seed plastids such as leucoplasts by fusing transit peptide gene sequences thereto will further enhance *in vivo* conditions for the biosynthesis of PHAs, especially P(3HB-co-3HV) copolymer, in plants.

Plastid Transformation for Expression of Enzymes Involved in PHA Biosynthesis

Alternatively, enzymes facilitating the biosynthesis of metabolites such as threonine, 2-oxobutyrate, propionyl-CoA, 3-ketovaleryl-CoA, 3-hydroxy-valeryl-CoA, and PHAs discussed herein can be expressed *in situ* in plastids by direct transformation of these organelles with appropriate recombinant expression constructs. Constructs and methods for stably transforming plastids of higher plants are well known in the art (Svab et al., 1990; Svab et al., 1993; Staub et al., 1993; Maliga et al., U.S. Patent No. 5,451,513; PCT International Publications WO 95/16783, WO 95/24492, and WO 95/24493). These methods generally rely on particle gun delivery of DNA containing a selectable marker in addition to introduced DNA sequences for expression, and targeting of the DNA to the plastid genome through homologous recombination.

Transformation of a wide variety of different monocots and dicots by particle gun bombardment is routine in the art (Hinchey et al., 1994; Walden and Wingender, 1995).

DNA constructs for plastid transformation generally comprise a targeting segment comprising flanking DNA sequences substantially homologous to a predetermined sequence of a plastid genome, which targeting segment enables insertion of DNA coding sequences of interest into the plastid genome by homologous recombination with said predetermined sequence; a selectable marker sequence, such as a sequence encoding a form of plastid 16S ribosomal RNA that is resistant to spectinomycin or streptomycin, or that encodes a protein which inactivates spectinomycin or streptomycin (such as the *aadA* gene), disposed within said targeting segment, wherein said selectable marker sequence confers a selectable phenotype upon plant cells, substantially all the plastids of which have been transformed with said DNA construct; and one or more DNA coding sequences of interest disposed within said targeting segment relative to said selectable marker sequence so as not to interfere with conferring of said selectable phenotype. In addition, plastid expression constructs also generally include a plastid promoter region and a transcription termination region capable of terminating transcription in a plant plastid, wherein said regions are operatively linked to the DNA coding sequences of interest.

A further refinement in chloroplast transformation/expression technology that facilitates control over the timing and tissue pattern of expression of introduced DNA coding sequences in plant plastid genomes has been described in PCT International

Publication WO 95/16783. This method involves the introduction into plant cells of constructs for nuclear transformation that provide for the expression of a viral single subunit RNA polymerase and targeting of this polymerase into the plastids via fusion to a plastid transit peptide. Transformation of plastids with DNA constructs comprising a viral single subunit RNA polymerase-specific promoter specific to the RNA polymerase expressed from the nuclear expression constructs operably linked to DNA coding sequences of interest permits control of the plastid expression constructs in a tissue and/or developmental specific manner in plants comprising both the nuclear polymerase construct and the plastid expression constructs. Expression of the nuclear RNA polymerase coding sequence can be placed under the control of either a constitutive promoter, or a tissue- or developmental stage-specific promoter, thereby extending this control to the plastid expression construct responsive to the plastid-targeted, nuclear-encoded viral RNA polymerase. The introduced DNA coding sequence can be a single encoding region, or may contain a number of consecutive encoding sequences to be expressed as an engineered or synthetic operon. The latter is especially attractive where, as in the present invention, it is desired to introduce multigene biochemical pathways into plastids. This approach is not practical using standard nuclear transformation techniques since each gene introduced therein must be engineered as a monocistron, including an encoded transit peptide and appropriate promoter and terminator signals. Individual gene expression levels may vary widely among different cistrons, thereby

possibly adversely affecting the overall biosynthetic process. This can be avoided by the chloroplast transformation approach.

Production of Transgenic Plants Comprising Genes for PHA Biosynthesis

Plant transformation vectors capable of delivering DNAs (genomic DNAs, plasmid DNAs, cDNAs, or synthetic DNAs) encoding PHA biosynthetic enzymes and other enzymes for optimizing substrate pools for PHA biosynthesis as discussed in Examples 1-7 herein can be easily designed. Various strategies can be employed to introduce these encoding DNAs to produce transgenic plants capable of biosynthesizing high levels of PHAs, including:

1. Transforming individual plants with an encoding DNA of interest. Two or more transgenic plants, each containing one of these DNAs, can then be grown and cross-pollinated so as to produce hybrid plants containing the two DNAs. The hybrid can then be crossed with the remaining transgenic plants in order to obtain a hybrid plant containing all DNAs of interest within its genome.
2. Sequentially transforming plants with plasmids containing each of the encoding DNAs of interest, respectively.
3. Simultaneously cotransforming plants with plasmids containing each of the encoding DNAs, respectively.

4. Transforming plants with a single plasmid containing two or more encoding DNAs of interest.

5. Transforming plants by a combination of any of the foregoing techniques in order to obtain a plant that expresses a desired combination of encoding DNAs of interest.

Traditional breeding of transformed plants produced according to any one of the foregoing methods by successive rounds of crossing can then be carried out to incorporate all the desired encoding DNAs in a single homozygous plant line (Nawrath et al., 1994; PCT International Publication WO 93/02187). Similar strategies can be employed to produce bacterial host cells engineered for optimal PHA production.

In methods 2 and 3, the use of vectors containing different selectable marker genes to facilitate selection of plants containing two or more different encoding DNAs is advantageous. Examples of useful selectable marker genes include those conferring resistance to kanamycin, hygromycin, sulphonamides, glyphosate, bialaphos, and phosphinothricin.

Stability of Transgene Expression

As several overexpressed enzymes may be required to produce optimal levels of substrates for copolymer formation, the phenomenon of co-suppression may influence transgene expression in transformed plants. Several strategies can be employed to avoid this potential problem (Finnegan and McElroy, 1994).

One commonly employed approach is to select and/or screen for transgenic plants that contain a single intact copy of the transgene or other encoding DNA

(Assaad et al., 1993; Vaucheret, 1993; McElroy and Brettell, 1994). *Agrobacterium*-mediated transformation technologies are preferred in this regard.

Inclusion of nuclear scaffold or matrix attachment regions (MAR) flanking a transgene has been shown to increase the level and reduce the variability associated with transgene expression in plants (Stief et al., 1989; Breyne et al., 1992; Allen et al., 1993; Mlynarova et al., 1994; Spiker and Thompson, 1996). Flanking a transgene or other encoding DNA with MAR elements may overcome problems associated with differential base composition between such transgenes or encoding DNAs and integrations sites, and/or the detrimental effects of sequences adjacent to transgene integration sites.

The use of enhancers from tissue-specific or developmentally-regulated genes may ensure that expression of a linked transgene or other encoding DNA occurs in the appropriately regulated manner.

The use of different combinations of promoters, plastid targeting sequences, and selectable markers for introduced transgenes or other encoding DNAs can avoid potential problems due to *trans*-inactivation in cases where pyramiding of different transgenes within a single plant is desired.

Finally, inactivation by co-suppression can be avoided by screening a number of independent transgenic plants to identify those that consistently overexpress particular introduced encoding DNAs (Register et al., 1994). Site-specific recombination in which the endogenous copy of a gene is replaced by the same gene, but with altered expression characteristics, should obviate this problem (Yoder and Goldsbrough, 1994).

Any of the foregoing methods, alone or in combination, can be employed in order to insure the stability of transgene expression in transgenic plants of the present invention.

Cloning of plastid pyruvate dehydrogenase complex and branched chain oxoacid dehydrogenase complex subunits and components

The present invention provides nucleotide sequences that encode the E1 α and E1 β subunits, and the E2 component, of the plastid pyruvate dehydrogenase complex, as well as the E1 α and E1 β subunits, and the E2 component, of the branched chain oxoacid dehydrogenase complex, of *Arabidopsis thaliana*. These sequences can be cloned by any appropriate method known in the art. For example, cDNA clones of known components of similar enzymes from other species can be utilized to screen a cDNA library from which the cDNA for the enzyme component is desired. Sources from which the plastid PDC E1 α and E1 β cDNAs can be obtained include the analogous enzyme-encoding cDNAs from the red alga *Porphyra purpurea*; for the E2 component of the plastid pyruvate dehydrogenase, the analogous enzyme gene from the cyanobacterium *Synechocystis* sp. can be used. The cDNA for the E1 α of a BCOADC can be isolated by identifying cDNAs which have significant homology to analogous tomato, human and bovine BCOADC E1 α sequences. Similarly, the E1 β and the E2 components of a BCOADC can be isolated by comparing the similarity of candidate sequences with the human and bovine BCOADC E1 β and E2 components, respectively. A cDNA library for the isolation of these components can be an expressed sequence tag library, for example one comprising cDNA from *Arabidopsis thaliana*.

The cloned cDNAs for the plastid PDC and the BCOADC components can be sequenced in order to determine the nucleotide sequence and deduce the amino acid sequence for these enzymes. The sequences of these cDNAs can be determined by any method known in the art. Methods for the determination of various portions of the sequenced cDNA, such as a plastid targeting sequence, are also well known in the art.

Engineering plants to produce propionyl-CoA in plastids

The production of the P(3HB-co-3HV) precursor propionyl-CoA in plastids requires the presence of two elements which are not present, or which are present at very low levels, in the plastids of wild-type plants: 2-oxobutyrate, and enzymes which will convert 2-oxobutyrate into propionyl-CoA.

As noted above, Gruys et al. (1998) discusses several methods for the production of 2-oxobutyrate in plastids. These include:

- Overexpression of threonine deaminase;
- Overexpression of aspartate kinase and threonine deaminase; and
- Overexpression of aspartate kinase, homoserine dehydrogenase, and threonine deaminase.

The overexpression of these enzymes can be accomplished through the transformation into plants of nucleotide sequences encoding these enzymes, operably linked to a plant promoter, such as the cauliflower mosaic virus (CaMV) 35s promoter, or any other promoter known in the art which causes overexpression of such enzymes in plants.

The expression of these and other enzymes in plastids can be achieved in at least two ways:

1. By transforming coding sequences for these enzymes directly into the plastid genome in such a way that they are incorporated into the plastid genome. Constructs and methods for stably transforming plastids of higher plants are well known in the art (for example, Svab et al., 1990; Svab et al., 1993; Staub et al., 1993; Maliga et al., U.S. Patent No. 5,451,513; PCT International Publications WO 95/16783, WO 95/24492, and WO 95/24493). These methods generally rely on particle gun delivery of DNA containing a selectable marker in addition to introduced DNA sequences for expression, and targeting of the DNA to the plastid genome through homologous recombination.

2. By creating a plant transformation vector comprising a coding sequence for the enzyme operably linked to a plastid targeting sequence, then transforming this vector into the plant. All of the enzymes discussed herein can be modified for plastid targeting by employing plant cell nuclear transformation constructs wherein DNA coding sequences of interest are fused to any of the available targeting peptide sequences capable of facilitating transport of the encoded enzymes into plant plastids, and driving expression by employing an appropriate promoter. Examples of plastid targeting peptides are provided in Table 1 and in von Heijne et al. (1991). The sequences that encode a targeting peptide region can be obtained, for example, from plant nuclear-encoded plastid proteins, such as the small subunit (SSU) of ribulose biphosphate carboxylase, plant fatty acid biosynthesis related genes including acyl carrier protein (ACP), stearoyl-ACP desaturase, β -ketoacyl-ACP synthase and acyl-ACP thioesterase, or LHCP II genes. The encoding sequence for a targeting peptide effective

in transport to plastids can include all or a portion of the encoding sequence for a particular targeting peptide, and can also contain portions of the mature protein encoding sequence associated with a particular targeting peptide. Numerous examples of targeting peptides that can be used to deliver target proteins into plastids exist, and the particular targeting peptide encoding sequences useful in the present invention are not critical as long as delivery into a plastid is obtained. Proteolytic processing within the plastid then produces the mature enzyme. This technique has proven successful not only with enzymes involved in PHA synthesis (Nawrath et al., 1994), but also with neomycin phosphotransferase II (NPT-II) and CP4 EPSPS (Padgett et al., 1995), for example.

Table 1. Examples of plastid proteins from various species with known plastid targeting sequences that can be used to target proteins to plastids

Chloroplast Targeting Peptides

Arabidopsis thaliana:

5-enolpyruvyl-shikimate-3-phosphate synthase
Rubisco activase
Rubisco small subunit
Tryptophan synthase

Brassica napus:

Acyl carrier protein
Plastid chaperonin-60

Pisum sativum:

Carbonic anhydrase
Chloroplast stromal HSP70
Glutamine synthetase
Rubisco small subunit

Reference: von Heijne, G.; Hirai, T.; Klosgen, R.B.; Steppuhn, J.; Bruce, B.; Keegstra, K.; Herrmann, R. (1991) CHLPEP-A database of chloroplast transit peptides. *Plant Molecular Biology Reporter* 9:104-126.

Engineering plants to produce poly(3-hydroxybutyrate-3-hydroxyvalerate) copolymer

Plants which produce P(3HB-co-3HV) can be created by engineering them to produce 2-oxobutyrate, to convert 2-oxobutyrate to propionyl-CoA, and to synthesize P(3HB-co-3HV) from propionyl-CoA and acetyl-CoA. Methods for producing plants which synthesize 2-oxobutyrate are discussed above. Such plants can be modified to convert 2-oxobutyrate to propionyl-CoA in the manner discussed below.

The nucleotide sequences of the BCOADC E1 α and E1 β subunits, and that of the E2 component, are provided herein as a means to effect the conversion of 2-oxobutyrate to propionyl-CoA in plastids containing the 2-oxobutyrate substrate. It is not necessary to provide the E3 component since the E3 components of all of the α -ketoacid dehydrogenase complexes are probably interchangeable. The E3 subunit already present in the plastid PDC thus almost certainly functions with plastid-targeted BCOADC subunits. The nucleotide sequences of the plastid PDC E1 α and E1 β subunits, and the E2 component, provide sources of plastid targeting sequences. These plastid PDC sequences can also be genetically manipulated to enhance their ability to convert 2-oxobutyrate to propionyl-CoA, as suggested by Gruys et al. (1998).

The nucleotide sequences encoding the BCOADC E1 α and E1 β subunits, and the E2 component, can be directly transformed into the plastid genome by the methods discussed above. Alternatively, the BCOADC E1 and E2 nucleotide sequences can be transformed into the plant nuclear genome, wherein the enzyme coding sequences are operably linked to a plastid targeting sequence by methods known in the art. See Example 7. Useful

plastid targeting sequences include those from the plastid PDC. These targeting sequences from *Arabidopsis thaliana* are disclosed in Examples 1 and 2, below.

As another alternative for utilizing a BCOADC for the conversion of 2-oxobutyrate to propionyl-CoA in plastids, a nucleotide sequence encoding the BCOADC E1 β subunit can be engineered to utilize the PDC E2 component which is already present in the plastids. The BCOADC E1 β subunit can be modified such that the native E2 binding region thereof is replaced with the E2 binding region of the plastid PDC E1 β subunit. The nucleotide sequences encoding the modified BCOADC E1 β subunit and the BCOADC E1 α subunit can also be operably linked to a plastid targeting sequence. The modified nucleotide sequences for these two subunits (α and β) of the BCOADC E1 component can then be inserted into plants by standard plant transformation methods, where they are translated in the cytoplasm. The enzymes are then transported to the plastid where they combine with the plastid PDC E2 and E3 components, and catalyze the conversion of 2-oxobutyrate to propionyl-CoA. See Example 6 below.

The conversion of propionyl-CoA and acetyl-CoA to P(3HB-co-3HV) requires a β -ketothiolase, a β -ketoacyl-CoA reductase, and a PHA synthase. Nucleotide sequences encoding these enzymes can be incorporated into the plastid genome directly, or into the nuclear genome, with operably linked plastid targeting sequences, utilizing the same well-known methods as previously discussed. Preferred β -ketothiolases are BktB and pAE65 from *A. eutrophus*, and *Zoogloea ramigera* β -ketothiolases "A" and "B", as disclosed in Gruys et al (1998). Preferred β -ketoacyl-CoA reductases and PHA

synthases include those from *A. eutrophus*, encoded by the *phbB* and *phbC* genes, respectively. However, the use of other β -ketothiolases which are able to utilize propionyl-CoA, and the use of other β -ketoacyl-CoA reductases and PHA synthases are within the scope of this invention. Included are those enzymes from, for example, *Alcaligenes faecalis*, *Aphanothece* sp., *Azotobacter vinelandii*, *Bacillus cereus*, *Bacillus megaterium*, *Beijerinckia indica*, *Derxia gummosa*, *Methylobacterium* sp., *Microcoleus* sp., *Nocardia corallina*, *Pseudomonas cepacia*, *Pseudomonas extorquens*, *Pseudomonas oleovorans*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, and *Thiocapsa pfennigii*.

P(3HB-co-3HV) Copolymer Composition

The P(3HB-co-3HV) copolymers of the present invention can comprise about 75-99% 3HB and about 1-25% 3HV based on the total weight of the polymer. More preferably, P(3HB-co-3HV) copolymers of the present invention comprise about 85-99% 3HB and about 1-15% 3HV. Even more preferably, such copolymers comprise about 90-99% 3HB and about 1-10% 3HV. P(3HB-co-3HV) copolymers comprising about 4%, about 8%, and about 12% 3HV on a weight basis possess properties that have made them commercially attractive for particular applications. One skilled in the art can modify P(3HB-co-3HV) copolymers of the present invention by physical or chemical means to produce copolymer derivatives having desirable properties different from those of the plant-produced copolymer.

Optimization of P(3HB-co-3HV) copolymer production by the methods discussed herein is expected to result in yields of copolymer in the range of from at least

about 1% to at least about 20% of the fresh weight of the plant tissue, organ, or structure in which it is produced.

The following examples illustrate the invention, but are not to be taken as limiting the various aspects of the invention so illustrated.

Conventional methods of gene isolation, molecular cloning, vector construction, etc., are well known in the art and are summarized in Sambrook et al., 1989, and Ausubel et al., 1989 and 1994. One skilled in the art can readily repeat the methods and reproduce the compositions described herein without undue experimentation. The various DNA sequences, fragments, etc., necessary for this purpose can be readily obtained as components of commercially available plasmids, or synthesized by well known methods, or are otherwise well known in the art and publicly available.

Example 1

Cloning and Sequencing cDNA Encoding the E1 α and E1 β Subunits of the Arabidopsis thaliana Plastid Pyruvate Dehydrogenase Complex

Expressed sequence tag (EST) clones (Reith et al., 1995) from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University were used to isolate full-length cDNAs for both the plastid E1 α and E1 β subunits from an *A. thaliana* cDNA library. Two clones (GenBank accessions T75600 and N65566) were identified as potentially encoding the plastid E1 α and E1 β subunits as follows.

Oligonucleotides were designed based on sequences common to *P. purpurea* *odpA* and *odpB* and the two *Arabidopsis* EST sequences and synthesized (all recited in the 5'-3' direction):

El α : 5' primer, CGGTACTCAAGTCTGACTCTGTCGTT (SEQ ID NO:7);

3' primer, CCTTCGAUAGGTTCCATCTCCGAAAAA (SEQ ID NO:8);

El β : 5' primer, CGGTACTCTTCGAGGCTCTTCAGGAA (SEQ ID NO:9);

3' primer, CCTTCGAUACGGGCCTTAGACCACT (SEQ ID NO:10).

The symbols denote restriction sites (t: Kpn I, and u: Hind III) added for subcloning. Thermal cycling was used to amplify cDNA fragments from *A. thaliana* using first strand cDNA. Thermal cycling reactions (50 μ l total volume) contained 10 mM Tris-HCl, pH 7.9, 1.25 mM MgCl₂, 25 μ M dNTPs, 5 units Taq polymerase (Promega, Madison, WI), 2 μ g *A. thaliana* first strand cDNA, and 10 ng of each primer. Thermal cycling was performed with a Perkin-Elmer model 480, with rapid ramp times set at 1°C/s. Cycling conditions were 94°C for 20 s, 50°C for 30 s, 72°C for 2 min with 6 s extensions each cycle and 30 rounds of cycling. Under these conditions, products containing 288 base pairs (El α) and 215 base pairs (El β) were obtained. The products were subcloned into pGEMT (Promega, Madison, WI) and sequenced to confirm their identity. Thermal cycling was also used to generate probes radiolabelled with (α^{32} P)-dCTP, using reaction mixtures identical to those previously described except for a 1000-fold reduction in the concentration of non-radioactive dCTP. Before use, the probes were desalted using Sephadex G-50 columns to remove unincorporated nucleotides. An *Arabidopsis* cDNA library (λ -PRL2, obtained from the ABRC) was plated at a density of 2.25×10^4 plaques per

plate for a total of 2.25×10^5 plaques. BioTrace NT nylon filters (Gelman, Ann Arbor, MI) were used for plaque-lifts and were processed according to the manufacturer's specifications. Hybridizations were performed according to *Current Protocols in Molecular Biology* (Ausubel et al., 1994). After three rounds of screening, 7 potential E1 α and 12 potential E1 β cDNA clones were isolated, ranging in size from 1100 to 1550 base pairs. Plaque-purified λ phage were treated according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD) in order to excise the pZL-1 recombinant clones.

DNA sequencing was performed using an ABI prism Model 377 sequencer, and analyzed using IntelliGenetics GeneWorks DNA analysis program version 2.5 on a Macintosh computer. Dye-deoxy terminating cycle sequencing reactions were carried out on both strands of full-length cDNA inserts and deletion fragments derived therefrom.

DNA isolation and Northern and Southern blotting were carried out according to *Current Protocols in Molecular Biology* (Sections 2.9.1, 4.3.1 and 4.9.1; Ausubel et al., 1994). RNA isolation was accomplished with the RNAGents total RNA isolation kit (Promega, Madison, WI). Northern blot prehybridization (3 h), hybridization (12 h), and 4 washes were done with 2.5 X SSPE (1X = 0.15 mM NaCl, 0.02 mM Na₂PO₄, 2 μ M EDTA, pH 7.4), 1% SDS, 1% non-fat dry milk, and 250 μ g/ml salmon sperm DNA at 68°C. Blots were exposed on Kodak X-OMAT/AR film (Rochester, New York) at -70°C with an intensifying screen.

Among the genes present in the *P. purpurea* plastome are two open reading frames, *odpA* and *odpB*, encoding proteins 32% identical to the *Arabidopsis*

mitochondrial E1 α and E1 β subunits (Grof et al., 1995; Leuthy et al., 1994; Leuthy et al., 1995). Attempts to use cloned mitochondrial PDC cDNAs as probes to identify plastid sequences have been unsuccessful. Based upon the *odpA* and *odpB* sequences, two EST clones (accessions T75600 and N65566) which appear to encode proteins more highly related to the *P. purpurea* *odpA* and *odpB* sequences than to the *Arabidopsis* mitochondrial sequences were used to isolate two cDNAs as potential E1 α and E1 β clones.

The nucleotide sequence of the *Arabidopsis* plastid PDC E1 α cDNA (Genbank Accession No. U80185) is shown in Appendix A and as SEQ ID NO:1. E1 α cDNA (1530 bp) has a 106 bp 5' untranslated region, a 1284 bp open reading frame encoding a polypeptide of 428 amino acids (Appendix B and SEQ ID NO:2), and a 140 bp 3' untranslated region. The nucleotide sequence of the *Arabidopsis* plastid PDH E1 β cDNA (Genbank Accession No. U80186) is shown in Appendix C and as SEQ ID NO:3. The E1 β cDNA (1441 bp) has a 6 bp 5' untranslated region, a 1218 bp open reading frame encoding a polypeptide of 406 amino acids (Appendix D and SEQ ID NO:4), and a 217 bp 3' untranslated region. The calculated molecular weight and isoelectric point values for the E1 α and E1 β polypeptides encoded by the open reading frames are 47,120 with a pI of 7.25, and 44,208 with a pI of 5.89, respectively. The deduced amino acid sequence for E1 α has 61%, and E1 β 68%, identity with *P. purpurea* *odpA* and *odpB*, respectively.

The first 68 residues of E1 α and the first 73 residues of E1 β exhibit characteristics of chloroplast targeting peptides but not those of mitochondrial targeting sequences (Gavel et al., 1990; von Heijne et al., 1989). To determine structural motifs of the targeting peptides, we used the GeneWorks (IntelliGenetics, Mountain View, CA) protein algorithm to identify possible α -helix and β -strands. Both plastid E1 α and E1 β have the potential to form amphiphilic β -strands consistent with plastid targeting sequences, but did not fit the amphiphilic α -helix which is characteristic of mitochondrial targeting sequences.

Tables 2 and 3 show the alignment of the deduced amino acid sequences of PDH E1 α and E1 β . Abbreviations are the same as in Fig 7. * indicates conserved, • non-conserved phosphorylation sites. ° indicates the conserved Cys 62 of the mature *H.s.* E1 α sequence.

Overall, there is 28% sequence identity between *Arabidopsis* plastid PDC E1 α and its mammalian counterparts. However, in specific regions, the degree of sequence conservation is much higher. The PDH component of PDC requires thiamine pyrophosphate (TPP) as a cofactor for decarboxylation of pyruvate (Patel et al., 1990). It has been reported that TPP binds to the E1 α subunit of mammalian PDH at a site containing a structural motif common to pyrophosphate-binding enzymes (Reed, 1974). A similar motif (50% identity with the bovine E1 α TPP-binding domain) is found in the *A. thaliana* plastid E1 α sequence at residues 160-213 (Table 2).

A highly conserved Cys residue (Cys 62 of mature human E1 α , Table 2) has been identified in eukaryotic PDH E1 α sequences, and it has been proposed that this

Cys is an essential component of the enzyme's active site (Ali et al., 1993). The *A. thaliana* plastid $E1\alpha$ sequence contains a similar motif, i.e. the same immediate flanking residues at 112-116, but the otherwise conserved Cys is replaced with a Val (Table 2).

Mitochondrial PDCs are regulated in part by reversible phosphorylation of three conserved Ser residues in the $E1\alpha$ sequence by a specific, complex-associated PDH-kinase (Reed, 1974). The Ser residues phosphorylated in mammalian mitochondrial PDH are also conserved in the plant mitochondrial (Luethy et al., 1995), yeast (Behal et al., 1989), and nematode (Johnson et al., 1992) amino acid sequences. However, while the plant mitochondria PDC is reversibly phosphorylated (Randall et al., 1989; Randall et al., 1996), all evidence to date indicates that plastid PDC activity is not regulated by phosphorylation (Camp et al., 1985). Despite this difference, the regulatory Ser residues and their flanking sequences are present in the plastid $E1\alpha$ sequence (Table 2). Korotchikina and Patel (1995) have reported the results from mutagenesis of these phosphorylation sites, and concluded that site one is closer to the active site or lies on the pathway to the main catalytic conformational change. This might explain why this region is so highly conserved. The amino acid-motif corresponding to phosphorylation site one in mitochondrial PDH sequences is present in the plastid polypeptide (Tyr 320-Pro 330 or Tyr 287-Pro 297 in the *H. s.* sequence, Table 2). Two of the four substitutions are by residues with conserved properties. The sequence of the plastid $E1\alpha$ corresponding to phosphorylation site two lacks a Ser and the region is dominated by five acidic and two

basic residues (Asp 329-Asp 339). The *Arabidopsis* plastid E1 α sequence contains a Ser at site 3 (Ala 259-Ala 267), but the flanking residues are dissimilar to the mammalian site 3 (Table 2). While two of the three Ser are in the appropriate positions, it is most likely then that plastid PDC is not regulated by phosphorylation due to the lack of plastid PDH-kinase (Camp et al., 1985).

Wexler et al. (1991) compared alignments of three PDH and three branched-chain α -keto acid dehydrogenase sequences. Among E1 β sequences, four regions of sequence conservation were observed. Region one, the proposed E2 interaction site, is present in the *Arabidopsis* plastid PDH E1 β sequence (Table 3). Conserved regions two and three share high homology with other decarboxylating enzymes, suggesting a role in decarboxylation of pyruvate (Wexler et al., 1991). A functional role has not yet been attributed to region four (Table 3). Eswaran et al. (1995) have described Arg 239 as being an essential residue near or at the active site of the bovine E1 β . This residue is conserved throughout the eukaryotic PDH sequences (e.g., Arg 269 of *H. s.* sequence in Table 3), and is present in the *A. thaliana* plastid E1 β sequence at position 318.

The genomic organization of *Arabidopsis* E1 α and E1 β was determined by Southern blot analysis. An E1 α cDNA probe hybridized to a single restriction fragment in each lane, suggesting one gene (Fig. 4A). An E1 β cDNA probe, on the other hand, hybridized to multiple fragments in a pattern consistent with the restriction digest of E1 β cDNA (data not shown). The Xba I lane

contained multiple hybridizing bands which could be due to a second gene or an intron containing an *Xba* I restriction site (Fig. 4B).

In order to evaluate expression of the *A. thaliana* plastid PDH genes, 10 μ g total RNA obtained from young leaves were resolved by formaldehyde gel electrophoresis. Northern blot analyses confirmed the expression of a single mRNA species of 1.65 kb for *El α* and 1.5 kb for *El β* (Figs. 5A and 5B).

The two cDNAs reported here have been identified as encoding plastid rather than mitochondrial proteins based on their high homology with the *P. purpurea* chloroplast genes, the presence of N-terminal sequences characteristic of plastid targeting peptides, and their relatively low homology with plant mitochondrial *E1* subunits (Grof et al., 1995; Leuthy et al., 1994; Leuthy et al., 1995). Assessments of the mature N-terminal sequences were based on homology with the mature *odp* and mitochondrial *E1* sequences.

The mature *A. thaliana* plastid *El α* and *El β* amino acid sequence have the highest homology (68%) with the *P. purpurea* chloroplast *odpA* and *odpB* sequences, respectively, but only 31 and 32% identity with the respective *A. thaliana* mitochondrial *E1* sequences (Tables 2 and 3). The homology with other eukaryotic mitochondrial *E1* sequences is lower yet. Additionally, a monoclonal antibody prepared against mitochondrial *El α* does not recognize chloroplastic *El α* (Luethy et al., 1995) nor does the monoclonal antibody recognize the recombinant plastid *El α* on immunoblots.

Dendrogram analyses show that *A. thaliana* plastid *E1*, *P. purpurea* chloroplast *odp*, and *Synechocystis* sp. (a cyanobacterium) *pdh* sequences segregate as a family distinct from mitochondrial and bacterial sequences

(Figs. 6A and 6B). A similar separation has also been shown for plastid and mitochondrial ribosomal RNA sequences (Palmer, 1992). The *A. thaliana* plastid cDNAs and *P. purpurea* *odp* genes are the only sequences reported thus far for plastid forms of PDH.

As additional cDNAs and genes for plastid and mitochondrial specific isozymes are determined, insight as to the lineage of plastid genes will be gained. Mitochondrial rRNA genes show convincing similarity to purple-photosynthetic bacterial rRNA sequences. In contrast, plastid rRNA has similarity with cyanobacterial rRNA. This relationship between plastids and cyanobacteria has also been noted for genes encoding the transcriptional and translational apparatus (Palmer, 1992). The new sequences reported here should contribute to understanding if the emergence of mitochondria and plastids was the result of single or multiple primary (i.e., eubacteria/eukaryotic) endosymbioses, or if secondary (i.e., eukaryotic/eukaryotic) endosymbioses led to the establishment of these organelles (Palmer, 1992).

Antibodies to the E1 α subunit of the plastid pyruvate dehydrogenase complex were generated by inserting the gel purified BamHI to HindIII fragment of the cDNA for E1 at the BamHI (5') to HindIII (3') cloning site of pET28a (Novagen). The recombinant clone was expressed, and the 5' end sequenced to ensure the correct reading frame. The recombinant protein was expressed using the above construct in *E. coli* strain BL21 (DE3) (Novagen). Growth conditions were as follows: A single colony was picked and cultured in 5 mL LB + 150 micrograms ampicillin overnight at 37 C shaking at 200 rpm. The 5ml culture was used to inoculate 500 mL LB + 150 microgram ampicillin and was

allowed to grow for 4 h. The culture was then induced using 0.1 mM IPTG and allowed to shake at 37 C for an additional 5 h. The culture was then centrifuged in a GSA rotor at 7,000 rpm to pellet cells. Cells were lysed in 6 M guanidinium HCl, 10 mM Tris pH 8.0 at room temperature. Cell debris was pelleted at 12,000 rpm in an SS-34 rotor for 20 min, and the recombinant protein was purified using Ni-NTA agarose. Rabbits were injected with 150 microgram of recombinant protein mixed 1:1 with complete adjuvant. A 30 day boost was given with the same protein preparation, at the same concentration. Ten days after the boost, the antibody titer was determined to be 1:80,000 against pea chloroplast stromal extract by immunoblot procedures.

It should be noted that the present invention encompasses not only the specific DNA sequences disclosed herein and the polypeptides encoded thereby, but also biologically functional equivalent nucleotide and amino acid sequences. The phrase "biologically functional equivalent nucleotide sequences" denotes DNAs and RNAs, including chromosomal DNA, plasmid DNA, cDNA, synthetic DNA, and mRNA nucleotide sequences, that encode polypeptides exhibiting the same or similar enzymatic activity as that of the enzyme polypeptides encoded by the sequences disclosed herein when assayed by standard enzymatic methods, or by complementation. Such biologically functional equivalent nucleotide sequences can encode polypeptides that contain a region or moiety exhibiting sequence similarity to the corresponding region or moiety of the present disclosed polypeptides.

One can isolate polypeptides useful in the present invention from various organisms based on homology or sequence identity. Although particular embodiments of

nucleotide sequences encoding the polypeptides disclosed herein are shown in the various SEQ IDs presented, it should be understood that other biologically functional equivalent forms of such polypeptide-encoding nucleic acids can be readily isolated using conventional DNA-DNA or DNA-RNA hybridization techniques. Thus, the present invention also includes nucleotide sequences that hybridize to any of the nucleic acid SEQ IDs and their complementary sequences presented herein, and that code on expression for polypeptides exhibiting the same or similar enzymatic activity as that of the presently disclosed polypeptides. Such nucleotide sequences preferably hybridize to the nucleic acid sequences presented herein or their complementary sequences under moderate to high stringency (see Sambrook et al., 1989). Exemplary conditions include initial hybridization in 6X SSC, 5X Denhardt's solution, 100 μ g/ml fish sperm DNA, 0.1% SDS, at 55°C for sufficient time to permit hybridization (e.g., several hours to overnight), followed by washing two times for 15 min each in 2X SSC, 0.1% SDS, at room temperature, and two times for 15 min each in 0.5-1X SSC, 0.1% SDS, at 55°C, followed by autoradiography. Typically, the nucleic acid molecule is capable of hybridizing when the hybridization mixture is washed at least one time in 0.1X SSC at 55°C, preferably at 60°C, and more preferably at 65°C.

The present invention also encompasses nucleotide sequences that hybridize under salt and temperature conditions equivalent to those described above to genomic DNA, plasmid DNA, cDNA, or synthetic DNA molecules that encode the same amino acid sequences as these nucleotide sequences, and genetically degenerate

forms thereof due to the degenerancy of the genetic code, and that code on expression for a polypeptide that has the same or similar enzymatic activity as that of the polypeptides disclosed herein.

Biologically functional equivalent nucleotide sequences of the present invention also include nucleotide sequences that encode conservative amino acid changes within the amino acid sequences of the present polypeptides, producing silent changes therein. Such nucleotide sequences thus contain corresponding base substitutions based upon the genetic code compared to the nucleotide sequences encoding the present polypeptides. Substitutes for an amino acid within the fundamental polypeptide amino acid sequences discussed herein can be selected from other members of the class to which the naturally occurring amino acid belongs. Amino acids can be divided into the following four groups: (1) acidic amino acids; (2) basic amino acids; (3) neutral polar amino acids; and (4) neutral non-polar amino acids. Representative amino acids within these various groups include, but are not limited to: (1) acidic (negatively charged) amino acids such as aspartic acid and glutamic acid; (2) basic (positively charged) amino acids such as arginine, histidine, and lysine; (3) neutral polar amino acids such as glycine, serine, threonine, cyteine, cystine, tyrosine, asparagine, and glutamine; and (4) neutral nonpolar (hydrophobic) amino acids such as alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine.

Conservative amino acid changes within the present polypeptide sequences can be made by substituting one amino acid within one of these groups with another amino acid within the same group. The encoding

nucleotide sequences (gene, plasmid DNA, cDNA, synthetic DNA, or mRNA) will thus have corresponding base substitutions, permitting them to code on expression for the biologically functional equivalent forms of the present polypeptides.

Useful biologically functional equivalent forms of the DNA sequences disclosed herein include DNAs comprising nucleotide sequences that exhibit a level of sequence identity to corresponding regions or moieties of these DNA sequences from 40% sequence identity, or from 60% sequence identity, or from 80% sequence identity, to 100% sequence identity to the DNAs encoding the presently disclosed polypeptides. However, regardless of the percent sequence identity of these nucleotide sequences, the encoded proteins would possess the same or similar enzymatic activity as the present polypeptides. Thus, biologically functional equivalent nucleotide sequences encompassed by the present invention include sequences having less than 40% sequence identity to any of the nucleic acid sequences presented herein, so long as they encode polypeptides having the same or similar enzymatic activity as the polypeptides disclosed herein.

Sequence identity can be determined using the "BestFit" or "Gap" programs of the Sequence Analysis Software Package, Genetics Computer Group, Inc., University of Wisconsin Biotechnology Center, Madison, WI 53711.

Due to the degeneracy of the genetic code, i.e., the existence of more than one codon for most of the amino acids naturally occurring in proteins, genetically degenerate DNA (and RNA) sequences that contain the same essential genetic information as the DNA sequences disclosed herein, and which encode the same amino acid

sequences as these DNA sequences, are encompassed by the present invention. Genetically degenerate forms of any of the other nucleic acid sequences discussed herein are encompassed by the present invention as well.

The alternative nucleotide sequences described above are considered to possess a biological function substantially equivalent to that of the polypeptide-encoding DNAs of the present invention if they encode polypeptides having enzymatic activity differing from that of any of the present polypeptides by about 30% or less, preferably by about 20% or less, and more preferably by about 10% or less when assayed *in vivo* by complementation or *in vitro* by the standard enzymatic assays.

Table 2

Alignment of the deduced amino acid sequences of PDC E1 α from various species. Abbreviations are the same as in Figure 6. * indicates conserved, . non-conserved phosphorylation sites. o indicates the conserved Cys 62 of the mature *H.s.* E1 α sequence.

Plastid A.t.	MATAFAPTKLTATVPLHGSHENRLLLPRLAPPSSFLGSTRSLRLRLNH	
50		
<i>P.purpurea</i>	-----	
<i>A.thaliana</i>	-----MALSRLLSSRSNIITRPFSAAPSRLLIS	
26		
<i>H.sapiens</i> II	-----MRKMLAAVSRVLSGASQKPASRVLVAS	
27		
<i>S.cerevisiae</i>	---MLAASFQRQPSQLVRGLGAVLRTPTRIGHVVRTMATLKTDDKAPEDI	
47		
<i>A.suum</i> I	-----MIFVFANIFKVPTVSPVSMIAISV	
23		
<i>M.capricolum</i>	-----MTYL	
4		
<i>B.subtilis</i>	-----MGVKTFQFPFAEQL	
14		
Consensus	-----	
50		

Motif 1

SNATRRSPVSVQEVVKEKQSTNNNTSLITKEEGLELYEDMILGRSPEDM	100
-----MSYPKKVELPLTNCQINLTKKLLVLVEDMLLGRNFEDM	40
TDTPITITETSLPPTAHLCDPPSRVSSESSQELLD-FFRTMALMRMEIA	75
RNFANDATFBIKKCDLHRLLEGPPVTTVLTRDGLKYRMMQTVRRMELK	77
EGSDTVQIELPESSPESYMLEPPDLSYETSATLLQMYKDMVIIRMEMA	97
RLASTTEATFQTKPFKLKHLDSGPDINHVHTKEDAVHYTQMLTIRRMESY	73
GKFDPLKNEKVCVLDKDGKVINPFLMPKI SDQILEAYKIMNLSRRQDIY	54
EKVAEQPPTFQILNEEGEVVNEEAMPELSDEQLKE-LMRRMVYTRILDQR	63
.....L..Y..M...RR.E..	100

o

CAQMYYRGKMFQFVHLYNGQEA VSTGFIKLITKSDSVVSTYRDH VHALSK	150
CAQMYYRGKMFQFVHLYNGQEA VSTGVIKLLDSKDYVCSTYRDH VHALSK	90
ADSLYKANVIRGFC HLYDGQEA VAI GMEAAITKKDAIITAYRDH CIFLGR	125
ADQLYKQKIIRGFC HLC DGQEACCGLEAGINPTDHLITAYRAHGFTFTR	127
CDALYKAKKIRGFC HLSVGQEAI AVGIEA NAITKLDSIITSYRCHGFTFMR	147
AGNLYKEKKVRGFC HLYSGQEACAVGT KAAMDAGDAAVTAYRCHGWYTLS	123
QNTMQRQGRLLSFLSSTGQEACEVAYI NALNKKTDHFVSGYRNNAAWLAM	104
STSLNRQGRLL-GFYAPTAGQEASQIASHFALEKEDFILPGYRDVQIITWH	112
...LY.....GF.HL..GQEA...G.....K.D.....YR.H.....	150

TPP-binding site

GV SARAVMSSELPFGKVTGCCRGQGGSMHMFSEHNMLGGFAFEGEIPVAT 200
 GVPFQNVMAELFGKETGCSRGRGSGSMHIFSAPHNFLGGFAFIEGIPVAT 140
 GGSLEHVFSELMGRQAGCSKGGKSGSMHIFYKKESSFYGGHGVGAQVPLGC 175
 GLSVREILAELETGRKGCCAKGKGGSMHMYAKN--FYGGNGIVGAQVPLGA 175
 GASVKAVLAELMGRAGVSYGKGGSMHLYAPG--FYGGNGIVGAQVPLGA 195
 GSSVAKVLCLETGRITGNVYGKGGSMHMYGEN--FYGGNGIVGAQVPLGT 171
 GQLVRNIMLYWIGNEAG-GKAPEG-VNCLFPN-----IVIGSQYSQAT 145
 GLPLYQAFPLFSRGHPHG-NQIPEG-VNVLFPQ-----IIIGAQYIQA 153

G.S...V..EL.G...G...G.GGSMH.....F.GG..I.GAQ.P... 200

PDH β binding site

GAAPSSKYRREVLKQDCD-DVTVAFFGDGTCTNNGQFFFECLNMAALYKLPI 249
 GAAPQSIYRQVLKEPGEELRVITACFFGDGTCTNNGQFFFECLNMAVLWKLP 190
 GIAFAQKYNKE---EA---VTFALYGDGAANQGQLFEALNISALWDLPA 218
 GIALACKYNGK---DE---VCLTYLGDGAANQGQIFAEYNMAALWKLP 218
 GLAFAHQYKNE---DA---CSFTLYLGDGASNGQGVFESFNMAKLWNLPV 238
 GIAFAMKYRKE---KN---VCITMFGDGATNQGQLFESMNMALWDLVP 214
 GIAFADKYRKT---GG---VVVTTTGDGGSSEGETYEAMNFAKLHEVPC 188
 GVALGLKMRGK---KA---VAITYTGDGGSQGDIFYEGINFAGAFKAPA 196

G.AFA.KYR.....V..T..GDG..NQCG.FE..NMA.LW.LP. 250

*3

IFVVENNLAIGMSHLRATSDPEIWKKGPAFGMPGVHVDGMDVLKREVA 299
 IFVVENNQWAIQMAHHRSSSIPEIHKKAEAFGLPGIEVDGMDVLAVRQVA 240
 ILCVENNHYGMGTAEWRAAKSPSYYKRGD-Y-VPLGLKVDGMDAFVVKQAC 266
 IPICCENNYGMGTSEVERAAASTDYKYRGD-F-IPGLRVVDGMDILCVREAT 266
 VPCENNNKYGMGTAAARSSAMTEYFPRGQ-Y-IPGLKVNVDGMDILAVYQAS 286
 LYVCENNNYGMGTAAARSSASTDYTRGD-Y-VPGIWDGMDVLAVRQAV 262
 IFVIEENNKWAISTARSEQTKSINFAVKGIATGPIPSIIVDGNLYLACIGVF 238
 IFVVQNNRFAISTPVEKQTVAKTLAQKAVAAGIPGQVDGMDPLAVYAAV 246

IFV.ENN....GTA..R.....K.G....PG..VDGMD.LAV..A. 300

*1 *2

KEAVTRARRGEGPTLVECTYRFRGHSLADPD-ELRDAAB-KAKYAARDP 347
 EKAVERARQGQPTLIEALTYRFRGHSLADPD-ELRSRQE-KEAWVARDP 288
 KFAQKHALE-KGPIILEMDTYRYHGHSMSPDGTSTYRTRDEISGVRQERDP 315
 RFAAAYCRSGKGPLMELOTYRYHGHSMSPDGPVSYRTREEIQEVRSKSDP 316
 KFAKDWCLSGKGPLVLEYETRYHGHSMSPDGTSTYRTRDEIQHMRSKNDP 336
 RWAKEWCNAGKPLMIEMATYRYHGHSMSPDGTSTYRTRREEVQEVKTRDP 312
 KEVVEYVRKNGNPVLVECDTYRLGAHSSSDNPDAIRPKGEPEEM-AKFPD 287
 KAARERAINGEGPTLIEALTYRFRGHSLADPD-ELRDAAB-KAKYAARDP 296

K.A.....G.GP.L.E..TYRY.GHSMSP...YR.R.E.....DP 350

IAALKKYLIENKLAKEAELKSIEKKIDELVEEAVEFADASPQPG--RSQL 395
 IKKLKXHLNDQIASSDELNDIQSSVKIDLEQSVFAMSSPEPN--ISEL 336
 IERIKKLVLSHDLATEKELKDMEKEIRKEVDDAIAKAKDCPMPE--PSEL 363
 IMLLKDRMVNSNLASVEELKEIDVEVRKEIEDAAQFATADPEPP--LEEL 364
 IAGLKMHLIDLGIATEAEVKAYDKSARKYVDEQVELADAAAPPEAKLSIL 386
 ITGFKDKLIVTAGLVTDEI KEIDKQVRKEIDAAVKQAHTDKESPVELMLT 362
 LIRLKQYLIDKKIWSDEQQAQLEAEQDKFVADEFWVEKNKNYDL-IDIF 336
 LVRFRKFLEAKGLWSEEEENNVIQAKEEIKEAIKKADETPKQK--VTDL 344

I..LK.....LA.E.E.K.....K....A...A...P.P.--...L 400

LENVFADPKGFGIGPDGRYRCEDPKFTEG-TAQV 428
 -----K-----RY-----LFADN----- 344
 FTVNVV--KGFG--TESFGPDRKEVKAS-LP-- 389
 GYHIYSSDPPF---EVRGANQWIKFKSVS--- 390
 FEDVYVKGTETPTLRGRIPEDTWDFFKQGFASRD 420
 DIYYNTPAQYVRCTTDEVLQKYLTSSEAVKALAK 396
 KYQYDKMDIFLEEQYKRAKEFFEKYEPESKEGGHH 370
 ISIMFE-ELPF-----NLKEQYEIYKEKESK-- 369

..... 434

Table 3

Alignment of the deduced amino acid sequences of PDC E1 β from various species. Abbreviations are the same as in Figure 6.

Plastid A.t.	MSSIIHGAGAATTTLSTFNSVDSKKLFVAPSRNTLSVRSQRYIVAGSDAS	
50		
P.purpurea	-----	
A.thaliana	-----	
H.sapiens	-----	
S.cerevisiae	-----	MFS
3		
A.suum	-----	
M.capricolum	-----	
B.subtilis	-----	
Consensus	-----	
50		

KSKFSGSLRVHSQKLI PNAVATKEADTSASTGHELLLFEALQEGLEEEM 100
 -----MSKVFMFDALRAATDEEM 18
 MLGILRQRAIDGASTLRRTRFALVSARSYAAGAKEMTVRDALNSAIDDEEM 50
 ---MAAVSGLVRRPLREVSGLLKRRFHNTAPAALQVTVRDALNQGMEDEL 47
 RLPTSLARNVARRAPTSFVRPSAAAAALRFSSTKTMVTVREALNSAMAEEL 53
 --MAVNGCMRLLRNGLTSACALEQSVRRLASGTLNVTVRDALNAALDEEI 48
 -----MAIINNIKAVTDALDCAM 18
 -----MAQMTMVQAITDALRIEL 18
 --.....T...AL..A.DEE. 100

Region 1

DRDPHVCMGEDVGHYGGSYKVTGLADKFGDLRVLDTPICENAFPTGMGI 150
 EKDLTVCVIGEDVGHYGGSYKVTGLHSHYGDRLVLDTPIAENSFTGMAI 68
 SADPKVFMGEEVGQYQYKIKTGLLEKYGPERVYDTPITEAGFTGIGV 100
 ERDEKVFLLGEEVAQYDGYKYSRGLWKKYGDKRIIDTPISEMGFAGIAV 97
 DRDDVFLIGEEVAQYNGAYKVSGLLDRFGERRVVDTPITEYGTGLAV 103
 KRDDRVLIGEEVAQYDGYKISKGLWKKYGDRIWDTPIITEMAIAGLSV 98
 QRDPNVIVFGEDVGTEGGVFRATQGLAVKFGNDRCFNAPISEAMFAGVGL 68
 KNDPNVLI FGEDVGNGGVFRATEGLQAEFGEDRVFDTPLAESGIGGLAI 68
 ..RD..V...GE.VG.Y.G.YK.TKGL..K.G..RV.DTPI.E..F.G... 150

GAAMTGLRPVIEGNNMGFLLAFNQISNNCGMLHYTSGGQFTIPVVIRGP 200
 GAAITGLRPIVEGMNMSFLLAFNQISNNAGMLRYTSGGNFTLPLVIRGP 118
 GAAYAGLKPVEFMFTFNFSMQAIDHIINSAAKSNYSAGQINVPVIFRGP 150
 GAAMAGLRPICEFMTFNFSMQAIDQVINSAAKTYMSGGQLQVPVIFRGP 147
 GAALKGLKPIVEFMTFNFSMQAIDHVVNSAAKTHYMSGGTQKQCMVFRGP 153
 GAAMNGLRPICEFMTFNFSMQGIDHIINSAAKAHYMSAGRFHVPIVFRGA 148

62

GMAMNGMKPVLEMQFEGLGLASLQNIFTNISMNRNRTGKYTAPMVIRMP 118
 GLALQGGFRPVPEIQFFGVYEVMSICQGMARIRYRTGGRYHMPITIRSP 118
 GAA..GLRP..E.M...F...A.D.I.N.AA...Y.SGG...P.V.RGP 200

Region 2

GGVGRQLGAEHSQRLESYFQSIPIGIQMVACSTPYNAKGLMKAIRSENBPV 250
 CGVGRQLGAEHSQRLEAYFOAIPGLKIVACSTPYNAKGLKSAIRDNNPV 168
 NGAAAGVGAQHSQCYAAWYASVPGGLKVLAPYSAEDARGLLKAAIRDPPDV 200
 NGASAGVAAQHSQCFAAWYGHCPGLKVVSPWNSEDAKGLIKSAIRDNNPV 197
 NGAAVGLGAQHSQDFSPWYGSIPGLKVLVPYSAEDARGLLKAAIRDNPV 203
 NGAAVGAQHSQDFTAWFMHCPGVKVVVPYDCEDARGLLKAAVRDDNPV 198
 MGGGIRALEHHSLEAEVYAHIPGVQIVCPSTPYDTKGLILAAIDSPDPV 168
 PGGGVHTPELHSDSLEGLVAQPGGLKVVIPSTPYDAKGLLISAIRDNDPV 168
 ..G.....A.HSQ...A.....PGLKVV.P....DAKGLLKA AIRD.NPV 250

ILFEHVLLYN---LKEKIPDEDIYCNLEBAEMVRPGEHITILTYSRMR 296
 VFVEHVLLYN---LQEEIPEDEYILPLDKAEVVRKGDITILTYSRMRH 214
 VFLENELLYGESFPISSEALDSSFCLPIGKAKIERBGKDVTIVTFSKMVG 250
 VVLENELMYGVPFEPFLPEAQSKDFLIPIGKAKIEROGTHITVVSHSRPVG 247
 VFLENELLYGESFPISEALSPEFTLPY-KAKIERBCTDISIVTYTRNV 252
 ICLNENELLYGMKPFVSPEAQSPDFVLPFGQAKIQPCGDITIVSLISGVD 248
 IVVEPTKLYR---AFKQEVDPDEHYIVPIGEGYKIQBGNDLTVVYGAQTV 215
 IFLEHLKLYR---SFRQEVPEGEYTIPIGKADIKREGKDITIIYAGAMVH 215
 ..LE..LLY.....E.....P.GKA.I.R.G.DITIVTYS..V. 300

Region 3

HVMQAAKTLVKN--GYDPEVIDIRSLKPFDLHTIGNSVKKTTHRVLIVEEC 344
 HVTEALPLLLND--GYDPEVLDLISLKLPLDIDSISVSVKKTTHRVLIVEEC 262
 FALKAAEKLAE--GISAEVINLRSIRPLDRATINASVRKTSRLVTVEEG 298
 HCLEAAAVLSKE--GVECEVINMRTIRPMDMETIEASVMKTNHLVTVEGG 295
 FSLAEAEILQKKY-GVSAEVINLRSIRPLDTEAIIKTVKKTNHLITVEST 301
 VSLHAADELAKS--GIDCEVINLRCVRPLDPQTOKDSVIKTKHLVTVEBSG 296
 DCQKAILALKETHPNATIDLIDLSIKFWDKMMVIESVKKTGRLLVVHEA 265
 ESLKAAAELEKE--GISAEVVDLRTVQPLDIETIIGSVKKTGRAIVVQEA 263
 ..L.AA..L...-G...EVI.LRS..PLD..TI..SV.KT.RL..VEE. 350

Region 4

MRTGGIGASLTAAINE-NFHDYLDAPVMCLSSQDVPTPYAGTLBEWTVVQ 393
 MKTAGIGAELIAQINE-HLFDDELDAVPVRLSSQDITPTPYNGSLEQATVIQ 311
 FPQHGVCACETCASVVE-ESFSYLDAPVRIAGADVPIPTANLERLALPQ 347
 WPQFGVGAETICARIMEGPAFNFLDAPAVRVVTGADVPMYAKILEDNSIPQ 345
 FPFSGVGAETIVAVQVMESEAFDYLDAPIQRVGTGADVPTPYAKELEDFAFPD 351
 WPNCGVGAETISARVTESDAFGYLDGPIRLRVGTGADVPMYAPQPLETAALPQ 346
 VKSFVSAAETIATVNE-ECFEYIKAPLSRCTGYDVITPFDGRG-EGYFQVN 313

QRQAGIAANVVAEINE-RAILSLEAPVLRVAAPDTVYPFAQA-ESVWLPN 311

....GVGAEI.A...E...F.YLDAP..R..G.DVP.PYA..LE....PQ 400

PAQIVTAVEQLCQ-----	406
PHQIIDAVKNIVNSSKTITT	331
IEDIVRASKRACYRSK----	363
VKDILFAIKKTLNI-----	359
TPTIVKAVKEVLSIE-----	366
PADVVKMVKKCLNVQ-----	361
PKKVLVKMQELLDKFK-----	329
FKDVIETAKKVMNF-----	325
...I..A.K.....-----	420

Example 2Cloning and Sequencing of a cDNAEncoding the *Arabidopsis thaliana**Dihydrolipoamide S-acetyltransferase (E2) Component*
of the Plastid Pyruvate Dehydrogenase Complex

A search of the *Arabidopsis* expressed sequence tagged (EST) database identified one *Arabidopsis thaliana* EST clone which has significant homology to the (cyanobacterial) *Synechocystis* sp. dihydrolipoamide acetyltransferase subunit, GenBank accession D90915. The *Arabidopsis* EST clone (GenBank accession W43179) was obtained from the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University, then used to screen an *Arabidopsis* λ PRL2 cDNA library (ABRC) for a full length clone as in Example 1. Two (approximately 1700 bp) clones assessed as full length, were identified and sequenced as in Example 1.

The plastid PDC E2 clone is 1709 bp in length (SEQ ID NO:5; GenBank accession AF066079) with a continuous open reading frame of 1440 bp encoding a protein of 480 amino acids (SEQ ID NO:6), with a deduced molecular mass of 52,400 daltons. The mature portion of the E2 component, without the chloroplast targeting peptide (see below), has a deduced molecular mass of 44,900 daltons. When subjected to SDS-PAGE electrophoresis, the full length and the mature plastid PDC E2 proteins ran slower than a globular protein of the same mass. These proteins appeared on SDS-PAGE to have molecular masses of 69,000 and 62,000, respectively. This slow migration on SDS-PAGE electrophoresis is consistent with the electrophoretic behavior of mitochondrial E2 components (Guest et al., 1985).

The mature part of the cDNA clone (coding for the catalytic region of the protein) was expressed in *E. coli* using the pET28c expression vector (Novagen, Madison,

WI). The recombinant protein (which includes a C-terminal six histidine tag) was purified under denaturing conditions by Ni-NTA affinity chromatography according to the manufacturer's instructions (Qiagen Inc., Chatsworth, CA). Polyclonal antibodies were raised to the recombinant protein in New Zealand White rabbits. These antibodies recognize the recombinant protein at a high dilution (1:100,000). In a analysis of an extract of purified pea chloroplasts, these antibodies recognized two proteins. One protein electrophoretically migrated at an apparent mass of 62,000, identical to the electrophoretic behavior of the mature plastid PDC E2 component. The other protein which was recognized by the anti-E2 antibodies had an electrophoretic mobility with an apparent mass of 76,000 daltons. This larger protein is likely due to mitochondrial contamination, since its apparent mass is equivalent to the mitochondrial E2 component.

The cDNAs for the *Arabidopsis thaliana* plastid E1 α , E1 β , and E2 were transcribed and translated *in vitro* using the TnT[™] transcription/translation system (Promega, Madison, WI) with the plasmid pZL1 (Life Technologies, Inc.) and the T7 promoter. Presenting the product to isolated pea chloroplasts resulted in ATP-dependent import into the plastid in a manner that protects it from protease action. This establishes that the cDNA sequences encode plastid targeting sequences. These targeting sequences are assessed to be the first 68 amino acids of the E1 α subunit (Appendix B and SEQ ID NO:2), the first 73 amino acids of the E1 β subunit (Appendix D and SEQ ID NO:4), and the first 54 amino acids of the E2 component (SEQ ID NO:6).

Example 3

Cloning and Sequencing of cDNA

Encoding the Arabidopsis thaliana El α Subunit of the Branched-Chain Oxoacid Dehydrogenase Complex

Selection of an *A. thaliana* expressed sequence tagged (EST) cDNA clone (Newman et al., 1994) was accomplished by searching the Arabidopsis EST database using the BLASTP program of the National Center for Biotechnology Information. One EST cDNA clone (GenBank accession N96041) was found to have significant homology to the tomato, human, and bovine BCOADC El α subunits, making it a candidate for the *A. thaliana* El α . This cDNA clone was obtained from the Arabidopsis Biological Resource Center at the Ohio State University. The clone was sequenced completely on both strands by subcloning restriction enzyme fragments of the clone and using two specific oligonucleotide primers designed from previously sequenced stretches. Sequencing was conducted by the DNA core facility at the University of Missouri, Columbia, MO on an ABI 377 instrument. The BCOADC El α cDNA clone is 1587 bp, with a 3' untranslated region of 165 bp (Appendix E and SEQ ID NO:11). The open reading frame encodes a protein of 472 amino acids (Appendix F and SEQ ID NO:12) with a deduced molecular mass of 53,363 daltons. We have not identified an initiating methionine/start codon, but alignment with the tomato, bovine, human and mouse sequences shows the clone is considerably longer than the mature coding region of these proteins.

The deduced amino acid sequence of the clone has significant homology to BCOADC El α sequences in the database: 56.8% identity with the tomato, 42% with the human, 40.7% with the bovine, and 41.6% with the mouse El α amino acid sequences. Though an initiating methionine was not identified, the N-terminus has properties similar to a mitochondrial targeting peptide.

The PSORT program (prediction of protein intracellular localization sites) suggests the mitochondrial matrix as the most probable destination of the *A. thaliana* E1 α protein. However, the amino acid sequence also contains an SKL motif close to the C-terminus which is indicative of peroxisomal localization, and this is the second most probable localization site determined by the PSORT program.

Ser₃₆₆ of the *A. thaliana* amino acid sequence is at a position which is conserved in all the above sequences. This site is a designated phosphorylation site for the mouse and bovine sequences. However, the second conserved Ser phosphorylation site in the animal sequences is replaced by a Pro in the tomato sequence and an Ala in the *A. thaliana* sequence (Appendix F and SEQ ID NO:12).

Example 4

Cloning and Sequencing of cDNA

Encoding the *Arabidopsis thaliana* E1 β Subunit of the Branched-Chain Oxoacid Dehydrogenase Complex

Selection of *Arabidopsis thaliana* expressed sequence tagged (EST) clones (Newman et al., 1994) was accomplished by searching the *Arabidopsis* EST database using the BLASTP PROGRAM of the National Center for Biotechnology Information. Two EST clones were found to have significant homology to the human and bovine branched-chain oxoacid dehydrogenase (BCOADC) E1 β subunit. These two clones (GenBank accessions T04217 and H37020) were identified as potentially encoding the *Arabidopsis thaliana* BCOADC E1 β subunits. We obtained these partial EST clones from the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University. One of these clones, GenBank accession T04217, was used to screen an *Arabidopsis* cDNA library for full length clones. The EST cDNAs were gel purified from low-melting

agarose and probes prepared by labeling with [α^{32} P]dATP using a random prime oligonucleotide labeling kit (Pharmacia, Piscataway, NJ). Probes were desalted using Sephadex G-50 chromatography to remove unincorporated nucleotides. An *Arabidopsis* cDNA library (λ -PRL2, obtained from the ABRC) was plated at a density of 2.9×10^4 plaques per plate for a total of 2.03×10^5 plaques. Biotrace NT nylon filters (Gelman, Ann Arbor, MI) were used for plaque-lifts and were processed according to the manufacturer's specifications. Prehybridization and hybridizations were performed according to *Current Protocols in Molecular Biology*, (Ausubel, et al., 1994). After three successive rounds of screening, 5 independent potential E1 β cDNA clones were isolated, ranging in size from 500 to 1400 bp. Two of the five cDNA clones were selected for sequencing. Plaque-purified λ phage were treated according to the manufacturer's instructions (GibcoBRL, Gaithersburg, MD) in order to excise the pZL-1 recombinant clones. The cDNA sequences were obtained by sequencing both strands of the cDNA clone (and deletion fragments derived therefrom) using the Dye-deoxy terminating cycle sequencing reactions and an ABI prism Model 377 sequencer, according to the manufacturer's instructions. Results from sequencing reactions were analyzed using IntelliGenetics GeneWorks DNA analysis program version 2.5 for Macintosh computers. Both cDNAs were identical. The BCOADC E1 β cDNA is 1319 bp (Appendix G and SEQ ID NO:13) and contains a 133 bp 5' untranslated region, an open reading frame of 1056 bp followed by 130 bp 3' untranslated region. The open reading frame encodes a protein with 352 deduced amino acids (Appendix H and SEQ ID NO:14) with a calculated mass of 37,810 Daltons.

Table 4 shows the alignment of the deduced amino acid sequences of various BCOADC E1 β subunits. "." indicates conserved amino acids; "-" indicates a gap

inserted to maximize homology. The deduced amino acid sequence is 59% identical to the mammalian BCOADC E1 β subunit (Table 4). The primary sequence contains no obvious organellar targeting information.

The cDNA was expressed in *E. coli* after insertion into the plasmid vector pMal (New England Biolabs). The purified protein was used to prepare polyclonal antibodies which recognize the recombinant protein.

Table 4

Alignment of the deduced amino acid sequences of various BCOADC E1 β subunits. Abbreviations are the same as in Figure 6.
 "." indicates conserved amino acids; "-" indicates a gap inserted to maximize homology.

A. t.	MAA-----LLG-RSC-----RKLSFPSLTHG-----ARR-
23	
Human	MAVVAAAAGWLLRLRAAGAEGHWRLPLGAGLARGFLHPAATVEDAAQRRQ
50	
Bovine	MAAVAAAFAGWLLRLRAAGADGPWRRLCGAGLSRGFLQSASAY-GAAQRRQ
49	
Consensus	MAAVAA.AGWLLRLRAAGA.G.WRRL.GAGL.RGFL..A...AAQRRQ
50	

V-----STETGKP--LNLYSAINQALHIALDTPRSYVFGEDEVGF 61
 VAHFTFQPDPEPREYGQTQKMNLFSVTSALDNSLAKDPTAVIFGEDVAF 100
 VAHFTFQPDPEPREYGQTQKMNLQAVTSALDNSLAKDPTAVIFGEDVAF 99
 VAHFTFQPDPEP.EYGQTQKMNLQAVTSALDNSLAKDPTAVIFGEDVAF 100

GGVFRCTTGLAERFGKNRVFNTPLCEQGIVGFGIGLAAMGNRAIVEIQFA 111
 GGVFRCTVGLRDYKGRVFNTPLECEQIVGFGIGIAVTGATAIAEIQFA 150
 GGVFRCTVGLRDYKGRVFNTPLECEQIVGFGIGIAVTGATAIAEIQFA 149
 GGVFRCTVGLRDYKGRVFNTPLECEQIVGFGIGIAVTGATAIAEIQFA 150

DYIYPAFDQIVNEAAKFRYRSGNQFNCGGLTIRAPYGA VGHGGHYHSQSP 161
 DYIYPAFDQIVNEAAKYRYSGLDFNCGSLTIRSPWGC VGHGALYHSQSP 200
 DYIYPAFDQIVNEAAKYRYSGLDFNCGSLTIRSPWGC VGHGALYHSQSP 199
 DYIYPAFDQIVNEAAKYRYSGLDFNCGSLTIRSPWGC VGHGALYHSQSP 200

EAFVCHVPGIKVVI PRSPREAGLLS CIRDNPVVFEPKWL YRAVEE 211
 EAFVCHVPGIKVVI PRSPFQAKGLLS CIEDKNPCIFFEPKIL YRAAAE 250
 EAFVCHVPGIKVVI PRSPFQAKGLLS CIEDKNPCIFFEPKIL YRAAVEQ 249
 EAFVCHVPGIKVVI PRSPFQAKGLLS CIEDKNPCIFFEPKIL YRAAVEE 250

VPEHDMIPLSAEVIREGNDITLVGWAQLTVMEQ-ACLD AEKEGISCE 260
VPIEPYNIPLSQAEVIQEGSDVTLVWGTQVHVIREVASM AKEKLGVSCE 300
VPVEPYNIPLSQAEVIQEGSDVTLVWGTQVHEIREVAAMAQEKLGVSCE 299

VP.EPYNIPLSQAEVIQEGSDVTLVWGTQVHVIREVA.MA.EKLGVSCE 300

LIDLKTLLPWDKETVEASVKKTGRLLISHEAPVTGGFGAEISATILERCF 310
VIDLRTIIPWDVDTICKSVIKSGRLLISHEAPLTGGFAEISSTVQEECF 350
VIDLRTILPWDVDTVCKSVIKTGRLLVSHEAPLTGGFAEISSTVQEQCF 349

VIDLRTILPWDVDTVCKSVIKTGRLLISHEAPLTGGFAEISSTVQE.CF 350

LKLEAPVSRVCGLDTPFPPLVFEPFYMPTKNKILDAIKSTVNY 352

LNLEAPISRVCGYDTPFPPIFEFFYIPDKWKCYDALRKMINY 392

LNLEAPISRVCGYDTPFPPIFEFFYIPDKWKCYDALRKMINY 391

LNLEAPISRVCGYDTPFPPIFEFFYIPDKWKCYDALRKMINY 392

Example 5Cloning and Sequencing of cDNAEncoding the *Arabidopsis thaliana*Dihydrolipoamide S-acyltransferase (E2) Component
of the Branched-Chain Oxoacid Dehydrogenase Complex

A search of the *Arabidopsis* expressed sequence tagged (EST) database identified two *Arabidopsis thaliana* EST clones which have significant homology to the bovine and human branched-chain dihydrolipoamide acyltransferase subunit. These clones (GenBank accessions T42996 and N37840) were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. Sequencing of the 5' ends of the two clones showed only one to be a branched-chain E2 sequence (the other contained vector sequence only). The branched-chain EST clone (GenBank accession T42996) was sequenced completely on both strands by subcloning of restriction enzyme derived fragments and by primer walking. Sequencing reactions and analysis were performed as in Example 1.

The clone (SEQ ID NO:15) is 1618 bp in length and contains an open reading frame of 1449 bp encoding a protein of 483 amino acids (SEQ ID NO:16) with a predicted molecular mass of 52,729 daltons. Part of the cDNA clone (coding for the lipoyl and subunit-binding domains, and part of the catalytic domain) was expressed in *E. coli* using the pET28a expression vector (Novagen, Madison, WI). The recombinant protein (which includes a C-terminal six histidine tag) was purified under denaturing conditions by Ni-NTA affinity chromatography according to the manufacturer's instructions (Qiagen Inc., Chatsworth, CA). Polyclonal antibodies were raised to the recombinant protein in New Zealand White rabbits. These antibodies recognize the recombinant protein at a high dilution (>1:100,000).

Example 6

Engineering Chimeric Branched Chain
Oxoacid Dehydrogenase Complex E1 α and E1 β Subunits
to Utilize the Plastid
Pyruvate Dehydrogenase Complex E2 and E3 Components
to Form a Hybrid Complex

The cDNA (or other encoding DNA) of the BCOADC E1 β subunit can be used to form a chimeric protein targeted to the plastid to utilize the plastid pyruvate dehydrogenase complex (PDC) E2 component to produce propionyl-CoA. The chimeric BCOADC E1 β subunit can be modified to comprise the E2 binding region of the plastid PDC E1 β subunit and a plastid targeting sequence. The thus modified BCOADC E1 β subunit can then be imported into the chloroplast, where it binds to the plastid PDC E2 component and, in conjunction with the plastid PDC E3 component, catalyzes the production of propionyl-CoA from 2-oxybutyrate. This leads to the production of the PHA precursor 3-hydroxyvaleryl-CoA, and consequently to biosynthesis of the PHA co-polymer poly(3HB-co-3HV) in plants that have been engineered to contain other enzymes necessary for biosynthesis of this copolymer, as discussed above.

The nucleotide sequence that encodes the BCOADC E1 β region 1 (the region or domain of the E1 β protein that binds the BCOADC E1 β component to the E2 core of the BCOADC complex [Wexler et al., 1991]) can be excised and replaced with the nucleotide sequence corresponding to the PDC E2 binding region from the plastid PDC E1 β subunit (Johnston et al., 1997; Luethy et al., 1994). The construct can be further engineered to comprise a plastid targeting sequence of another plastid protein such as the Rubisco small subunit (Table 1) (von Heijne et al., 1991), or to comprise the plastid targeting sequence of the plastid PDC E1 β subunit described by Johnston et al. (1997). See Figure 7B.

Chimeric fusions of plastid targeting sequences and the BCOADC E1 α and E1 β subunits can be generated by amplifying fragments of DNA coding for the regions involved. Chloroplast targeting peptides from each of the plastid PDC E1 subunits (PDC E1 α and E1 β) (Johnston et al., 1997) can be amplified from the original cDNAs (SEQ ID NOS 1 and 3). Similarly, the mature portions of the BCOADC E1 α and E1 β subunits can be amplified from their cDNAs (SEQ ID NOS 11 and 13). A unique restriction site can be included in the primer design to permit ligation of the chloroplast targeting peptides in-frame with the mature portions of the BCOADC E1 α and E1 β subunits.

To produce a BCOADC E1 β chimera that can associate with the PDC E2 subunit, one can modify the BCOADC E1 β subunit to include the plastid PDC E1 β targeting peptide along with the plastid PDC E1 β E2 binding region. In the final construct, the sequence for the E2 binding region follows (i.e., is 3' to) the sequence for the targeting peptide, so that the chimeric BCOADC E1 β protein contains approximately one-third plastid PDC E1 β presequence (for example, amino acid residues 1 through 146 of SEQ ID NO:4) and the remainder consists of the BCOADC E1 β subunit (for example, amino acid residues 94 through 352 of SEQ ID NO:14). The PDC E1 β chloroplast targeting peptide and plastid PDC E2 binding region of the PDC E1 β subunit can be amplified from the plastid PDC E1 β cDNA (SEQ ID NO:4) using the following gene specific primer (SEQ ID NO:28) and a commercially available primer (e.g. M13/pUC forward primer, available from e.g. Stratagene, La Jolla, CA).

Forward oligonucleotide: 5' GGGCCC CATATG TCTTCGATAATC 3' (SEQ ID NO:28). Nucleotides 7 through 21 are preceded by an NdeI enzyme site.

The mature part of the BCOADC E1 β sequence (excluding the native BCOADC E2 binding site) can be amplified from the cDNA of SEQ ID NO:13 using the following gene specific primers:
Forward oligonucleotide: 5' GGGCCC ACCGGT TTGGCATTGGTCTA 3' (SEQ ID NO:24). Nucleotides 406 through 423 are preceded by an AgeI enzyme site.

Reverse oligonucleotide: 5' GGGCCC GAATTC TCATTACTAGTAATTCAC AGT 3' (SEQ ID NO:25). Nucleotides 1177 through 1191 are preceded by an EcoRI enzyme site.

The resulting truncated BCOADC E1 β sequence can be ligated to the plastid PDC E1 β sequence using the AgeI enzyme site already present in the plastid PDC sequence at a convenient position (amino acid residue 146). The above primers can be utilized to produce DNA fragments useful in joining the noted regions of the plastid PDC and BCOADC E1 β sequences without any introduced or substituted amino acids (Figure 7B).

To produce a BCOADC E1 α chimera that can be targeted to a plastid, a chloroplast targeting peptide, for example the chloroplast targeting peptide from the plastid PDC E1 α subunit (Johnston et al., 1997) (corresponding to amino acid residues 1 through 68) can be attached 5' to the mature portion of the BCOADC E1 α subunit. A DNA fragment corresponding to the plastid targeting peptide can be amplified from the original PDC E1 α cDNA (SEQ ID NO:1) using the following gene specific primers (SEQ ID NO:29 and SEQ ID NO:30):

Forward primer: 5' GGGCCC CCATGG CGACGGCTTTCGCT 3' (SEQ ID NO:29). Nucleotides 107 to 124 are preceded by an NcoI enzyme site.

Reverse primer: 5' GGGCCC TGATCA TATTATTGGTGGATTGCTT 3' (SEQ ID NO:30). Nucleotides 311 to 328 are preceded by a BclI enzyme site.

The entire mature coding region of the BCOADC E1 α subunit can then be excised from the cDNA (SEQ ID NO:11) using convenient restriction enzyme sites, BclI at nucleotides 195 through 200, and XbaI at nucleotides 1424 through 1429. This includes the 3' stop codon.

The restriction enzyme fragments generated from both the plastid PDC and BCOADC E1 α sequences can then be ligated together and subcloned into an appropriate vector (e.g. pZL1, Life Technologies Inc., Gaithersburg, MD). The BclI site used to ligate the two sequences introduces a single His residue between the plastid PDC E1 β targeting peptide and the BCOADC E1 α mature region.

The consequence of this addition can be determined experimentally to assess its impact, if any, on import and processing of the BCOADC E1 α subunit, and on assembly of the hybrid BCOADC E1 complex.

An alternative approach to ligating the plastid PDC and BCOADC E1 α sequences using the BclI site is to use a NotI site in its place in the design of the reverse oligonucleotide for the plastid targeting peptide, as follows (SEQ ID NO:19):

Plastid PDC E1 α reverse primer: 5' GGGCCC GCGGCCGC ATTATTGGTGGATTGCTT 3' (SEQ ID NO:19). Nucleotides 311 through 328 are preceded by a NotI enzyme site.

The coding region for the mature BCOADC E1 α protein (Appendix F and SEQ ID NO:12) can then be amplified from the cDNA (SEQ ID NO:11) using the following gene-specific primers:

Forward primer: 5' GGGCCC GCGGCCGC TGATCATTTGGTTCAGCAG 3' (SEQ ID NO:20). Nucleotides 195 through 213 are preceded by a NotI enzyme site.

Reverse primer: 5' GGGCCC GTCGAC TCAAACATGAAAGCCAGG 3' (SEQ ID NO:21). Nucleotides 1405 through 1422 are preceded by a SalI enzyme site and includes the stop codon.

Ligation of the two resulting sequences using the NotI enzyme site will introduce three Ala residues between them, which would overcome the introduction of a charged residue (His) using the BclI site described above.

To confirm the ability of the chimeric BCOADC E1 α and E1 β proteins to be imported into chloroplasts, the DNA encoding these chimeric proteins can be subcloned into a transcription vector such as pZL1 (Life Technologies Inc., Gaithersburg, MD) with the T7 promoter. The chimeric proteins are then transcribed/translated *in vitro*, for example using the TnT[™] transcription/translation system (Life Technologies Inc.), and import assays with isolated chloroplasts can be performed. This is a reliable assay to test the import and assembly of the chimeric proteins.

Experimental results have established that *in vitro* imported plastid PDC E1 α and E1 β subunit proteins associate to form the plastid pyruvate dehydrogenase heterotetramer within the chloroplast matrix, and that this heterotetramer associates with imported PDC E2 subunits (Randall et al., unpublished).

To obtain constitutive expression of the chimeric proteins in plants, their coding regions are preferably fused to the CaMV 35S promoter sequence. For dicotyledonous plants, the use of the pZP200 binary vector, for *Agrobacterium* transformation, is preferred.

The chimeric nucleic acids disclosed above are used to transform *Arabidopsis thaliana* or other plants by various methods well known in the art. As one alternative, the BCOADC E1 α -chimeric construct comprising the plastid PDC E1 α targeting sequence is used to produce transformed plants that are then crossed with plants that have been transformed with the BCOADC E1 β -chimeric construct containing the plastid PDC E1 β subunit targeting sequence and E2 component binding region.

As another alternative, a compound construct containing both the plastid-targeted BCOADC E1 α -chimera and the plastid-targeted BCOADC E1 β -chimera containing the PDC E1 β E2 binding region is constructed in the form of a mega plasmid and used to transform plants by standard protocols for expression of both subunit chimeras simultaneously (Figure 7D). This can be achieved by including a stop signal at the 3' end of the BCOADC E1 α chimeric sequence and a NOS transcription termination sequence. In order to obtain co-expression of the two chimeric sequences, a second CaMV 35S promoter sequence can be placed 3' to the transcription termination sequence of the plastid-targeted BCOADC E1 α chimeric coding sequence. This second promoter sequence can in turn be followed by the sequence coding for the BCOADC E1 β chimera. This creates a mega plasmid or compound construct coding for both the BCOADC E1 α and β subunit chimeras (Figure 7D).

The BCOADC E1 α and β subunit chimeras thus targeted to the plastid bind to the plastid PDC E2 component (E2 components form the core of the complexes to which the E1 and E3 components bind). Since the chimeric BCOADC E1 β subunit comprises the plastid PDC E1 β E2 binding domain, a hybrid complex is formed. This hybrid complex is designed to have an enhanced ability to utilize 2-oxobutyrate as substrate in order to produce propionyl-CoA for 3-HV biosynthesis. Transgenic plants containing this hybrid complex can then be crossed by standard protocols with plants having enhanced ability to generate 2-oxobutyrate in the plastid compartment produced as described, for example, in Gruys et al. (1998).

Example 7Targeting the BCOADC E1 α , E1 β , and E2 components
to the Plastid to Form a Hybrid Complex
with the Plastid PDC E3 Component

DNAs encoding the BCOADC E1 α and β subunits and E2 component can be fused with plastid targeting sequences to direct importation of these proteins into the plastid to enhance propionyl-CoA production from 2-oxobutyrate. In this method, constructs of the BCOADC E1 α and β subunits, the BCOADC E2 component, and, if desired, the BCOADC E3 subunit, can be made with plastid targeting sequences, for example with plastid targeting sequences of the plastid pyruvate dehydrogenase complex (PDC) E1 α and β subunits (Johnston et al., 1997) or the plastid PDC E2 component. See Figures 7A, 7C, and 7E. These constructs can be used to transform plants individually (followed by genetic crossing to combine the necessary components from each plant) or together to direct the desired BCOADC components to the plastid. The BCOADC E1 α -chimera is as described above in Example 6. The BCOADC E1 β -chimera containing the PDC E1 β E2 binding region is also described in Example 6. When the plastid-targeted BCOADC E2 chimera is also employed (see below), the E2 binding region of the BCOADC E1 β subunit need not be replaced with the plastid PDC E1 β subunit E2 binding region. Instead, only the plastid PDC E1 β targeting peptide is attached to the mature portion of the BCOADC E1 β subunit (still retaining the native binding site for the BCOADC E2 component) (Figure 7E). This can be achieved by amplifying the appropriate regions of the PDC and BCOADC E1 β cDNA sequences or other functionally equivalent DNA sequences. That portion of the cDNA coding for the plastid targeting peptide of the PDC E1 β (amino acids 1 through 97) can be amplified from the cDNA (SEQ ID NO.:3) using the following gene specific primers.

This amplified fragment includes a portion of the linker region between the targeting peptide and the E2-binding region.

Forward oligonucleotide: 5' GGGCCC CATATG TCTTCGATAATC 3' (SEQ ID NO:22). Nucleotides 7 through 21 are preceded by an NdeI enzyme site.

Reverse oligonucleotide: 5' GGGCCC CTCGAG ACCTTCCTGAAGAGC 3' (SEQ ID NO:23). Nucleotides 277 through 297 are preceded by an XhoI enzyme site.

The mature portion of the BCOADC E1 β sequence (including the native BCOADC E2 binding region), i.e., amino acid residues 45 through 349, can be amplified from the cDNA of SEQ ID NO:13 using the following gene specific primers:

Forward oligonucleotide: 5' GGGCCC CTCGAG ATCGCTTTGGACACC 3' (SEQ ID NO:31). Nucleotides 262 through 277 are preceded by an XhoI enzyme site.

Reverse oligonucleotide: 5' GGGCCC GAATTC TCATTACTAGTAATTAC AGT 3' (SEQ ID NO:25). Nucleotides 1177 through 1191 are preceded by an EcoRI enzyme site.

Use of the foregoing oligonucleotide primers allows the joining of the appropriate plastid PDC and BCOADC E1 β sequences without any introduced or substituted amino acids (Figure 7E). As disclosed in Example 6, the resulting DNA can be subcloned into a transcription vector to test import and assembly prior to transformation of *Arabidopsis* or other plants (or prior to the construction of a mega plasmid for co-expression, cf. Figure 7D).

Further to the above, a chimera comprising the plastid targeting sequence (nucleotides 59-232) of the plastid PDC E2 (dihydrolipoamide acetyltransferase) component and the sequence for the mature BCOADC dihydrolipoamide acyltransferase (E2) subunit can be constructed. The N-terminus of the BCOADC E2 subunit can be replaced with the chloroplast targeting peptide from

the plastid PDC E2 subunit. In this case, the native E2 binding domain of the BCOADC E1 β subunit need not be replaced with the E2 binding domain of the plastid PDC E1 β subunit as described in Example 6. Only the plastid PDC E2 targeting peptide is needed because the BCOADC E2 component which is imported into the plastid will naturally associate with the BCOADC E1 β subunit.

The plastid targeting sequence can be amplified from the plastid PDC E2 cDNA of SEQ ID NO:5 using the following gene-specific primers:

Forward primer: 5' GGGCCC CATATG GCGGTTTCTTCT 3' (SEQ ID NO:26). Nucleotides 59 through 73 are preceded by an NdeI enzyme site.

Reverse primer; 5' GGGCCC CCATGGC AATTTCAGGATTCTT 3' (SEQ ID NO:27). Nucleotides 218 through 232 are preceded by an NcoI enzyme site.

The region coding for the mature portion of the BCOADC E2 protein can be excised from the cDNA (SEQ ID NO.:15) using convenient restriction enzymes (NcoI and NotI). This DNA fragment is then ligated in-frame with the PDC E2 plastid targeting peptide using the common NcoI enzyme site (Figure 7C). As described in Example 6, the import and assembly of this chimeric E2 subunit can be examined by *in vitro* import assays. Efficient import of the BCOADC E2 protein into isolated pea chloroplasts and formation of a complex with both the endogenous PDC heterotetramer and imported BCOADC E1 α -E1 β heterotetramer can be determined.

The plastid-targeted branched-chain oxoacid dehydrogenase complex components utilize any 2-oxobutyrate (α -ketobutyrate) produced in the plastid to make propionyl CoA, which in turn is a substrate for the enzymes producing polyhydroxy-alkanoic acids (PHAs).

As previously indicated, it appears to be unnecessary to prepare a plastid-targeted construct for the BCOADC E3 component since the E3 components of all of the mitochondrial α -ketoacid dehydrogenase complexes appear to be interchangeable. The PDC E3 component already present in the plastid should function with the plastid-targeted BCOADC E1 α , E1 β , and E2 subunits. If desired, one can, for example, place a plastid targeting sequence on the mitochondrial E3 component in place of the first 31 amino acids of the mitochondrial PDC E3 reported by Turner et al. (1992) (GenBank accession number X2995), corresponding to the first 72 nucleotides of that particular cDNA. This is done by standard protocols well known to those skilled in the art.

As discussed above, the plastid is capable of PHA biosynthesis when the appropriate enzymes are present in the plant (Poirier et al., 1992; Nawrath et al., 1994). Targeting BCOADC subunits and components to this organelle as described in Examples 6 and 7 herein further enhances ability of plants to biosynthesize the 3HB-co-3HV copolymer.

The invention being thus described, it will be obvious that the same can be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the present invention, and all such modifications and equivalents as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Johnston, Mark L.
Miernyk, Jan A.
Luethy, Michael H.
Mooney, Brian P.
- (ii) TITLE OF INVENTION: USE OF DNA ENCODING PLASTID PYRUVATE
DEHYDROGENASE AND BRANCHED CHAIN OXOACID DEHYDROGENASE
COMPONENTS TO ENHANCE POLYHYDROXYALKANOATE BIOSYNTHESIS IN
PLANTS
- (iii) NUMBER OF SEQUENCES: 32
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- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
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 - (C) CLASSIFICATION:
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1541 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Arabidopsis thaliana*

(I) ORGANELLE: Chloroplast

(vii) IMMEDIATE SOURCE:

(B) CLONE: Pyruvate dehydrogenase complex E1alpha

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCACGGCTCC GCATCTCTTG TTCTCTCCGC CCATCTCTGC TCTCTTTAT
TTTCCAGAA 60

AGTTTTTTTT TTTTTTCCG AATTCGGTA ATCTATTGG GGTTCATTG GATAGCAATG
120

GCGACGGCTT TCGCTCCAC TAAGCTCACT GCCACGGTTC CTCTGCATGG
ATCCCATGAG 180

AATCGTCTCT TGCTCCCGAT CCGATTGGCT CCTCTTCTT CTTTCTCGG
ATCCACCCGT 240

TCCCTCTCCC TTCGCAGACT CAATCACTCC AACGCCACCC GTCGATCTCC
CGTCGTCTCT 300

GTCCAGGAAG TTGTCAAGGA GAAGCAATCC ACCAATAATA CCAGCCTGT
GATAACCAAA 360

GAGGAAGGAT TGGAGTTGTA TGAAGATATG ATACTAGGTA GATCTTTTCA
AGACATGTGT 420

GCTCAAATGT ATTACCGAGG CAAGATGTTT GGTTTTGTTC ACTTGTAACA
TGGCCAAGAG 480

GCTGTTTCTA CTGGCTTTAT CAAGCTCCTT ACCAAGTCTG ACTCTGTCTG
TAGTACCTAC 540

CGTGACCATG TCCATGCCCT CAGCAAAGGT GTCTCTGCTC GTGCTGTTAT
GAGCGAGCTC 600

TTCGGCAAGG TTA CTGGATG CTGCAGAGGC CAAGGTGGAT CCATGCACAT
GTTCTCCAAA 660

GAACACAACA TGCTTGTTGG CTTTGCTTTT ATTGGTGAAG GCATTCTCTG
CGCCACTGGT 720

GCTGCCTTTA GCTCCAAGTA CAGGAGGGAA GTCTTGAAAC AGGATTGTGA
TGATGTCACT 780

GTGCGCTTTT TCGGAGATGG AACTTGTAAC AACGGACAGT TCTTCGAGTG
TCTCAACATG 840

GCTGCTCTCT ATAAACTGCC TATTATCTTT GTGTGCGAGA ATAACTTGTG
GGCCATTGGG 900

ATGTCTCACT TGAGAGCCAC TTCTGACCCC GAGATTGGA AGAAAGGTCC

TGCATTGGG 960

ATGCTGGTG TTCATGTGA CGGTATGGAT GTCITGAAGG TCAGGGAAGT
CGCTAAAGAA 1020

GCTGTCACTA GAGCTAGAAG AGGAGAAGGT CCAACCTTGG TTGAATGTGA
GACTTATAGA 1080

TTCAAGAGGAC ACTCCTTGGC TGATCCCGAT GAGCTCCGTG ATGCTGCTGA
GAAAGCCAAA 1140

TACGCGGCTA GAGACCCAAT CGCAGCATTG AAGAAGTATT TGATAGAGAA
CAAGCTTGCA 1200

AAGGAAGCAG AGCTAAAGTC AATAGAGAAA AAGATAGACG AGTTGGTGGA
GGAAGCGTT 1260

GAGTTTGCAG ACGCTAGTCC ACAGCCCGGT CGCAGTCAGT TGCTAGAGAA
TGTGTTTGCT 1320

GATCCAAAAG GATTTGGAAAT TGGACCTGAT GGACGGTACA GATGTGAGGA
CCCAGTTT 1380

ACCGAAGGCA CAGCTCAAGT CTGAGAAGAC AAGTTTAACC ATAAGCTGTC
TACTGTCTCT 1440

TCGATGTTTC TATATATCTT ATTAAGTTAA ATGCTACAGA GAATCAGTTT
GAATCATTTG 1500

CACTTTTTCG TTAaaaaaaaa aaaaaaaaaa aaaaaaaaaa A

1541

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 428 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Arabidopsis thaliana*
- (I) ORGANELLE: Chloroplast

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Pyruvate dehydrogenase complex Elalpha

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..68
- (D) OTHER INFORMATION: /note= "Targeting site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Thr Ala Phe Ala Pro Thr Lys Leu Thr Ala Thr Val Pro Leu
 1 5 10 15
 His Gly Ser His Glu Asn Arg Leu Leu Leu Pro Ile Arg Leu Ala Pro
 20 25 30
 Pro Ser Ser Phe Leu Gly Ser Thr Arg Ser Leu Ser Leu Arg Arg Leu
 35 40 45
 Asn His Ser Asn Ala Thr Arg Arg Ser Pro Val Val Ser Val Gln Glu
 50 55 60
 Val Val Lys Glu Lys Gln Ser Thr Asn Asn Thr Ser Leu Leu Ile Thr
 65 70 75 80
 Lys Glu Glu Gly Leu Glu Leu Tyr Glu Asp Met Ile Leu Gly Arg Ser
 85 90 95
 Phe Glu Asp Met Cys Ala Gln Met Tyr Tyr Arg Gly Lys Met Phe Gly
 100 105 110
 Phe Val His Leu Tyr Asn Gly Gln Glu Ala Val Ser Thr Gly Phe Ile
 115 120 125
 Lys Leu Leu Thr Lys Ser Asp Ser Val Val Ser Thr Tyr Arg Asp His
 130 135 140
 Val His Ala Leu Ser Lys Gly Val Ser Ala Arg Ala Val Met Ser Glu
 145 150 155 160
 Leu Phe Gly Lys Val Thr Gly Cys Cys Arg Gly Gln Gly Ser Met
 165 170 175
 His Met Phe Ser Lys Glu His Asn Met Leu Gly Gly Phe Ala Phe Ile
 180 185 190
 Gly Glu Gly Ile Pro Val Ala Thr Gly Ala Ala Phe Ser Ser Lys Tyr
 195 200 205
 Arg Arg Glu Val Leu Lys Gln Asp Cys Asp Asp Val Thr Val Ala Phe
 210 215 220
 Phe Gly Asp Gly Thr Cys Asn Asn Gly Gln Phe Phe Glu Cys Leu Asn
 225 230 235 240
 Met Ala Ala Leu Tyr Lys Leu Pro Ile Ile Phe Val Val Glu Asn Asn
 245 250 255
 Leu Trp Ala Ile Gly Met Ser His Leu Arg Ala Thr Ser Asp Pro Glu
 260 265 270
 Ile Trp Lys Lys Gly Pro Ala Phe Gly Met Pro Gly Val His Val Asp
 275 280 285
 Gly Met Asp Val Leu Lys Val Arg Glu Val Ala Lys Glu Ala Val Thr
 290 295 300
 Arg Ala Arg Arg Gly Glu Gly Pro Thr Leu Val Glu Cys Glu Thr Tyr

305 310 315 320
 Arg Phe Arg Gly His Ser Leu Ala Asp Pro Asp Glu Leu Arg Asp Ala
 325 330 335
 Ala Glu Lys Ala Lys Tyr Ala Ala Arg Asp Pro Ile Ala Ala Leu Lys
 340 345 350
 Lys Tyr Leu Ile Glu Asn Lys Leu Ala Lys Glu Ala Glu Leu Lys Ser
 355 360 365
 Ile Glu Lys Lys Ile Asp Glu Leu Val Glu Glu Ala Val Glu Phe Ala
 370 375 380
 Asp Ala Ser Pro Gln Pro Gly Arg Ser Gln Leu Leu Glu Asn Val Phe
 385 390 395 400
 Ala Asp Pro Lys Gly Phe Gly Ile Gly Pro Asp Gly Arg Tyr Arg Cys
 405 410 415
 Glu Asp Pro Lys Phe Thr Glu Thr Ala Gln Val
 420 425

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1441 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Arabidopsis thaliana*

(I) ORGANELLE: Chloroplast

(vii) IMMEDIATE SOURCE:

(B) CLONE: Pyruvate dehydrogenase complex E1beta

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAAAAAATGT CTTCGATAAT CCATGGAGCT GGAGCTGCTA CGACGACGTT
 ATCGACGTTT 60

AATTCCGTCG ATTCCAAGAA ACTCTTCGTT GTCCTTCTC GCACAAATCT
 TTCAGTGAGG 120

AGCCAGAGAT ATATAGTGGC TGGATCTGAT GCGAGTAAGA AGAGCTTTGG
 TTCTGGACTT 180

AGAGTTCGTC ACTCTCAGAA ATTGATTCCA AATGCTGTTG CGACGAAGGA
 GCGGATACG 240

TCTGCGAGCA CTGGACATGA ACTATTGCTT TTCGAGGCTC TTCAGGAAGG
TCTGGAAGAA 300

GAGATGGACA GAGATCCACA TGTATGTGTT ATGGGTGAAG ATGTTGGCCA
TTACGGAGGT 360

TCCTACAAGG TAACCAAGG CCTTGCTGAT AAATTTGGTG ACCTCAGGGT
TCTCGACACT 420

CCTATTTGTG AAAATGCATT CACCGGTATG GGCATTGGAG CTGCCATGAC
TGGTCTAAGA 480

CCCGTTATTG AAGGTATGAA CATGGGTTTC CTCCTCTCG CCTCAACCA
AATCTCCAAC 540

AACTGTGGAA TGCTTACTA CACATCCGGT GGTCAGTTTA CGATCCCGGT
TGTCATCCGT 600

GGACCTGGTG GAGTGGGACG CCAGCTTGTT GCTGAGCATT CACAGAGGTT
AGAATCTTAC 660

TTTCAGTCCA TCCCTGGGAT CCAGATGGTT GCTTGCTCAA CTCCTACAA
CGCCAAAGGG 720

TTGATGAAAG CCGCAATAAG AAGCGAGAAC CCTGTGATTC TGTTCAACAA
CGTGCTGCTT 780

TACAATCTCA AGGAGAAAAT CCCGGATGAA GATTACATCT GTAACCTTGA
AGAAGCTGAG 840

ATGGTCAGAC CTGGCGAGCA CATTACCATC CTCACTTACT CGCGAATGAG
GTACCATGTG 900

ATGCAGGCAG CAAAACTCT GGTGAACAAA GGGTATGACC CCGAGGTTAT
CGACATCAGG 960

TCACTGAAAC CGTTCGACCT TCACACAATT GGAACCTCGG TGAAGAAAAAC
ACATCGGGTT 1020

TTGATCGTGG AGGAGTGTAT GAGAACCGGT GGGATTGGGG CAAGTCTTAC
AGCTGCCATC 1080

AACGAGAACT TTCATGACTA CTTAGATGCT CCGGTGATGT GTTTATCTTC
TCAAGACGTT 1140

CCTACACCTT ACGCTGGTAC ACTGGAGGAG TGGACCGTGG TTCAACCGGC
TCAGATCGTG 1200

ACCGCTGTGC AGCAGCTTTG CCAGTAAATT CATATTTATC CGATGAACCA
TTATTTATCA 1260

TTTACCTCTC CATTTCTTTT CTCTGTAGCT TAGTCTTAA AGAATTTGTC
TAAGATGGTT 1320

TGTTTTTGTG AAAGTTTGTG TCCTTTGTG TGCTTTTAA TATGGTTTGT AACTCAGAAT
1380

GTTTGTGTG TAATTTTATC TCCCACTTTC TTTTAAAAA AAAAAAAAAA

AAAAAAAAA 1440

A

1441

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 406 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Arabidopsis thaliana*
- (I) ORGANELLE: Chloroplast

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Pyruvate dehydrogenase complex E1beta

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..73
- (D) OTHER INFORMATION: /note= "Targeting peptide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Ser Ser Ile Ile His Gly Ala Gly Ala Ala Thr Thr Thr Leu Ser
 1           5           10           15

Thr Phe Asn Ser Val Asp Ser Lys Lys Leu Phe Val Ala Pro Ser Arg
      20           25           30

Thr Asn Leu Ser Val Arg Ser Gln Arg Tyr Ile Val Ala Gly Ser Asp
      35           40           45

Ala Ser Lys Lys Ser Phe Gly Ser Gly Leu Arg Val Arg His Ser Gln
      50           55           60

Lys Leu Ile Pro Asn Ala Val Ala Thr Lys Glu Ala Asp Thr Ser Ala
      65           70           75           80

Ser Thr Gly His Glu Leu Leu Leu Phe Glu Ala Leu Gln Glu Gly Leu
      85           90           95

Glu Glu Glu Met Asp Arg Asp Pro His Val Cys Val Met Gly Glu Asp
      100          105          110

Val Gly His Tyr Gly Gly Ser Tyr Lys Val Thr Lys Gly Leu Ala Asp
      115          120          125

Lys Phe Gly Asp Leu Arg Val Leu Asp Thr Pro Ile Cys Glu Asn Ala
      130          135          140

Phe Thr Gly Met Gly Ile Gly Ala Ala Met Thr Gly Leu Arg Pro Val
      145          150          155          160

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Ile Glu Gly Met Asn Met Gly Phe Leu Leu Ala Phe Asn Gln Ile
165 170 175

Ser Asn Asn Cys Gly Met Leu His Tyr Thr Ser Gly Gly Gln Phe Thr
180 185 190

Ile Pro Val Val Ile Arg Gly Pro Gly Gly Val Gly Arg Gln Leu Gly
195 200 205

Ala Glu His Ser Gln Arg Leu Glu Ser Tyr Phe Gln Ser Ile Pro Gly
210 215 220

Ile Gln Met Val Ala Cys Ser Thr Pro Tyr Asn Ala Lys Gly Leu Met
225 230 235 240

Lys Ala Ala Ile Arg Ser Glu Asn Pro Val Ile Leu Phe Glu His Val
245 250 255

Leu Leu Tyr Asn Leu Lys Glu Lys Ile Pro Asp Glu Asp Tyr Ile Cys
260 265 270

Asn Leu Glu Glu Ala Glu Met Val Arg Pro Gly Glu His Ile Thr Ile
275 280 285

Leu Thr Tyr Ser Arg Met Arg Tyr His Val Met Gln Ala Ala Lys Thr
290 295 300

Leu Val Asn Lys Gly Tyr Asp Pro Glu Val Ile Asp Ile Arg Ser Leu
305 310 315 320

Lys Pro Phe Asp Leu His Thr Ile Gly Asn Ser Val Lys Tyr Thr His
325 330 335

Arg Val Leu Ile Val Glu Glu Cys Met Arg Thr Gly Gly Ile Gly Ala
340 345 350

Ser Leu Thr Ala Ala Ile Asn Glu Asn Phe His Asp Tyr Leu Asp Ala
355 360 365

Pro Val Met Cys Leu Ser Ser Gln Asp Val Pro Thr Pro Tyr Ala Gly
370 375 380

Thr Leu Glu Glu Trp Thr Val Val Gln Pro Ala Gln Ile Val Thr Ala
385 390 395 400

Val Glu Gln Leu Cys Gln
405

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1708 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Arabidopsis thaliana*

(I) ORGANELLE: Chloroplast

(vii) IMMEDIATE SOURCE:

(B) CLONE: Pyruvate dehydrogenase complex E2

(ix) FEATURE:

(A) NAME/KEY: mRNA

(B) LOCATION: 59..1498

(D) OTHER INFORMATION: /function= "Open Reading Frame"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGTCCACTTC ACTCTCTCTA AACTCTCTCT CAGATCTCTC TCTCTCTGTG
ATTCAACAAT 60

GGCGGTTTCT TCTTCTTCGT TTCTATCGAC AGCTTCACTA ACCAATTCCA
AATCCAACAT 120

TTTATTCGCT TCCTCAGTAT CCCCATCCCT CCGCAGCGTC GTTTCCCGT
CCACGACTCC 180

GGCGACTTCT CACCGTCGTT CAATGACGGT CCGATCTAAG ATTCTGAAA
TTTTCATGCC 240

GGCGTTATCA TCAACCATGA CGGAAGGCAA AATCGTGTC TGGATCAAAA
CAGAAGGCGA 300

GAAACTCGCC AAGGGAGAGA GTGTTGTGGT TGTGAATCT GATAAAGCCC
ATATGGATGT 360

AGAAACGTTT TACGATGGTT ATCTTGCTGC GATTGTCGTC GGAGAAGGTG
AAACAGCTCC 420

GGTTGGTGCT GCGATTGGAT TGTTAGCTGA GACTGAAGCT GAGATCGAAG
AAGCTAAGAG 480

TAAAGCCGCT TCGAAATCTT CTCTTCTGT GGCTGAGGCT GTCGTTCCAT
CTCTCTCTCC 540

GGTACTTCT TCTCCTGCTC CGCGGATTGC TCAACCGGCT CCGGTGACGG
CAGTATCAGA 600

TGGTCCGAGG AAGACTGTTG CGACGCCGTA TGCTAAGAAG CTGCTAAAC
AACACAAGGT 660

TGATATTGAA TCCGTTGCTG GAACTGGACC ATTCGGTAGG ATTACGGCTT
CTGATGTGGA 720

GACGGCGGCT GGAATTGCTC CGTCCAAATC CTCCATCGCA CCACCGCCTC
CTCCTCCACC 780

TCCGGTGACG GCTAAAGCAA CCACCACTAA TTGCTCTCT CTGTTACCTG
ATTCAAGCAT 840

TGTTCTTTTC ACAGCAATGC AATCTGCAGT ATCTAAGAAC ATGATTGAGA

GTCTCTCTGT 900

TCTACATTC CGTGTGGTT ATCTGTGAA CACTGACGCT CTTGATGCAC
TTTACGAGAA 960GGTGAAGCCA AAGGGTGTA CAATGACAGC TTTATTAGCT AAAGCTGCAG
GGATGCCCTT 1020GGCTCAGCAT CCTGTGGTGA ACGCTAGCTG CAAAGACGGG AAGAGTTTAA
GTTACAATAG 1080TAGCATTAAC ATTGCAGTGG CGGTTCTAT CAATGGTGGC CTGATTACGC
CTGTTCTACA 1140AGATGCAGAT AAGTTGGATT TGTACTTGTT ATCTCAAAAA TGGAAAAGAGC
TGGTGGGAA 1200AGCTAGAAGC AAGCAACTTC AACCCCATGA ATACAACCTT GGAACTTTAA
CTTTATCGAA 1260TCTCGGTATG TTTGGAGTGG ATAGATTGA CGCTATTCTT CCGCCAGGAC
AGGGTGCTAT 1320TATGGCTGTT GGAGCGTCAA AGCCAACTGT AGTTGCTGAT AAGGATGGAT
TCTTCAGTGT 1380AAAAACACA ATGCTGGTGA ATGTGACTGC AGATCATCGC ATTGTGTATG
GAGCTGACTT 1440GGCTGCTTTT CTCCAAACCT TTGCAAAGAT CATTGAGAAT CCAGATAGTT
TGACCTTATA 1500AGACGCCAAG CGAAGACGAG AAGTCAAAAA CAGTTTCAA AATTCTGTAG
CCAAATTTTT 1560CCCAAGTAAA TTTTAAACC TCAATGTTCT TGGGCTTCCC CAACTTCTTT
TGATCTTTT 1620CCTCACTGG GTTGTACCGG TATTGGTTT CAAGAATCAC CATTTTGGGG
TTTAAACAAA 1680

TAATTTCCAA CCAAAAAAAAA AAAAAAAAAA

1708

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 480 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Arabidopsis thaliana*
- (I) ORGANELLE: Chloroplast

(vii) IMMEDIATE SOURCE:

(B) CLONE: Pyruvate dehydrogenase complex E2

(ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION: 1..54

(D) OTHER INFORMATION: /note= "Targeting region"

(ix) FEATURE:

(A) NAME/KEY: Domain

(B) LOCATION: 58..127

(D) OTHER INFORMATION: /note= "Lipoyl domain"

(ix) FEATURE:

(A) NAME/KEY: Binding-site

(B) LOCATION: 189..221

(D) OTHER INFORMATION: /note= "Subunit binding domain"

(ix) FEATURE:

(A) NAME/KEY: Active-site

(B) LOCATION: 260..480

(D) OTHER INFORMATION: /note= "Inner catalytic domain"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Ala Val Ser Ser Ser Ser Phe Leu Ser Thr Ala Ser Leu Thr Asn
1 5 10 15

Ser Lys Ser Asn Ile Ser Phe Ala Ser Ser Val Ser Pro Ser Leu Arg
20 25 30

Ser Val Val Phe Arg Ser Thr Thr Pro Ala Thr Ser His Arg Arg Ser
35 40 45

Met Thr Val Arg Ser Lys Ile Arg Glu Ile Phe Met Pro Ala Leu Ser
50 55 60

Ser Thr Met Thr Glu Gly Lys Ile Val Ser Trp Ile Lys Thr Glu Gly
65 70 75 80

Glu Lys Leu Ala Lys Gly Glu Ser Val Val Val Val Glu Ser Asp Lys
85 90 95

Ala Asp Met Asp Val Glu Thr Phe Tyr Asp Gly Tyr Leu Ala Ala Ile
100 105 110

Val Val Gly Glu Gly Glu Thr Ala Pro Val Gly Ala Ala Ile Gly Leu
115 120 125

Leu Ala Glu Thr Glu Ala Glu Ile Glu Glu Ala Lys Ser Lys Ala Ala
130 135 140

Ser Lys Ser Ser Ser Ser Val Ala Glu Ala Val Val Pro Ser Pro Pro
145 150 155 160

Pro Val Thr Ser Ser Pro Ala Pro Ala Ile Ala Gln Pro Ala Pro Val
165 170 175

Thr Ala Val Ser Asp Gly Pro Arg Lys Thr Val Ala Thr Pro Tyr Ala
180 185 190

Lys Lys Leu Ala Lys Gln His Lys Val Asp Ile Glu Ser Val Ala Gly
195 200 205

Thr Gly Pro Phe Gly Arg Ile Thr Ala Ser Asp Val Glu Thr Ala Ala
210 215 220

Gly Ile Ala Pro Ser Lys Ser Ser Ile Ala Pro Pro Pro Pro Pro
225 230 235 240

Pro Pro Val Thr Ala Lys Ala Thr Thr Thr Asn Leu Pro Pro Leu Leu
245 250 255

Pro Asp Ser Ser Ile Val Pro Phe Thr Ala Met Gln Ser Ala Val Ser
260 265 270

Lys Asn Met Ile Glu Ser Leu Ser Val Pro Thr Phe Arg Val Gly Tyr
275 280 285

Pro Val Asn Thr Asp Ala Leu Asp Ala Leu Tyr Glu Lys Val Lys Pro
290 295 300

Lys Gly Val Thr Met Thr Ala Leu Leu Ala Lys Ala Ala Gly Met Ala
305 310 315 320

Leu Ala Gln His Pro Val Val Asn Ala Ser Cys Lys Asp Gly Lys Ser
325 330 335

Phe Ser Tyr Asn Ser Ser Ile Asn Ile Ala Val Ala Val Ala Ile Asn
340 345 350

Gly Gly Leu Ile Thr Pro Val Leu Gln Asp Ala Asp Lys Leu Asp Leu
355 360 365

Tyr Leu Leu Ser Gln Lys Trp Lys Glu Leu Val Gly Lys Ala Arg Ser
370 375 380

Lys Gln Leu Gln Pro His Glu Tyr Asn Ser Gly Thr Phe Thr Leu Ser
385 390 395 400

Asn Leu Gly Met Phe Gly Val Asp Arg Phe Asp Ala Ile Leu Pro Pro
405 410 415

Gly Gln Gly Ala Ile Met Ala Val Gly Ala Ser Lys Pro Thr Val Val
420 425 430

Ala Asp Lys Asp Gly Phe Phe Ser Val Lys Asn Thr Met Leu Val Asn
435 440 445

Val Thr Ala Asp His Arg Ile Val Tyr Gly Ala Asp Leu Ala Ala Phe
450 455 460

Leu Gln Thr Phe Ala Lys Ile Ile Glu Asn Pro Asp Ser Leu Thr Leu
465 470 475 480

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Pyruvate dehydrogenase complex Elalpha 5' primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGGTACCAAG TCTGACTCTG TCGTT

25

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Pyruvate dehydrogenase complex Elalpha 3' primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCTTCGAAGG TTCCATCTCC GAAAAA

26

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Pyruvate dehydrogenase complex Elbeta 5' primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGGTACCTTC GAGGCTCTTC AGGAA

25

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Pyruvate dehydrogenase complex E1beta 3' primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCTTCGAACG GGCCTTAGAC CAGT

24

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1587 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Arabidopsis thaliana

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Branched chain oxoacid dehydrogenase complex E1alpha

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGGCGATCTG GTTTGCTAGA TCCTAAACCC TTGTTTCTAG CTGAGACAT
AATCTAAATT 60TGTGACAAT TCTCATAAAA CGTGATTACT CTCATCGTCC CATCTTCTAT
ACAACCTTCTC 120AGTTATCTTC AACGGCGTAT TTGAGTCCCT TCGGTAGCCT CCGTCATGAG
TCTACGGCCG 180TGGAGACACA GGCTGATCAT TTGGTTCAGC AGATTGATGA AGTCGATGCC
CAGGAAGTGG 240ATTTCACAGG AGGCAAGTC GGTACACAT CGGAGATGAA ATTCATACCG
GAATCATCTT 300

CAAGGAGGAT TCCATGTTAC CGGGTTCTTG ACGAAGACGG ACGAATCATC

CCCGATAGCG 360
ATTTTATTCC GGTGAGTGAG AAACCTGCTG TTAGAATGTA CGAACAAATG
GCGACGCTAC 420
AAGTAATGGA TCACATCTTC TACGAAGCTC AACGTCAAGG AAGAATATCT
TTTTATCTTA 480
CTTCCGTGGG AGAAGAAGCC ATTAACATCG CTTACGAGC TGCTCTCAGT
CCTGACGACG 540
TCGTTTTACC TCAGTACCGA GAACCTGGAG TTCTTTTGTG GCGTGGCTTC
ACGTTGGAGG 600
AGTTTGCTAA TCAGTGTTTT GGGACAAAG CTGATTATGG CAAAGGCAGA
CAAATGCCAA 660
TTCAATACGG TTCCAATCGT CITAATTACT TCACTATCTC CTCTCCAATT
GCCACGCAAC 720
TTCTCAAGC TGCTGGAGTT GGTATTCTT TGAATAAGG CAAGAAGAAT
GCTTGACTG 780
TTACATTCAT CGGAGATGGT GGCACAAGCG AGGGAGATT TCACGCCGGA
TTGAATTTTG 840
CGGCCGTAAT GGAAGCTCCG GTTGTTTGA TATGTCGGAA CAACGGTTGG
GCGATTAGTA 900
CTCATATCTC AGAACAGTTT AGAAGTGATG GAATAGTTGT GAAAGGTCAA
GCTTACGGTA 960
TCCCGAAGCA TCCCGTGTGG GACGGTACCG ATGCACTTGC GGTTTATAGT
GCTGTACGCT 1020
CAGCTCGAGA AATGGCTGTA ACAGAACAAA GACCTGTCTT CATTGAGATG
ATGACATATA 1080
GAGTAGGACA TCATTCTACA TCAGATGATT CAACTAAGTA CAGGGCGGCG
GATGAAATCC 1140
AGTACTGGAA AATGTCGAGA AACCTGTGA ATAGATTCG GAAATGGGTC
GAAGATAACG 1200
GATGGTGGAG TGAGGAAGAT GAATCCAAGC TAAGATCTAA CGCAAGAAAA
CAGCTTCTGC 1260
AAGCGATTCA GGCTCGCGAG AAGTGGGAGA AACCAACCAATT GACAGAGTTG
TTTAACGATG 1320
TATATGATGT TAAACCGAAG AACCTAGAAG AGCAAGAACT TGGTTTGAAG
GAATTAGTAA 1380
AGAAACAACC TCAAGATTAT CCTCCTGGCT TTCATGTTG AATCTAGAGG
AAGTGTGG 1440
TTAAATACC TCGCGGACCG CGAATTCGAT ATCAAGCTTC TCATTGCAGA
CTATTTATAT 1500

TGTCCACGTA TCGAATAGTA ATCAAGTATC AATGTAGAGA CCAGCATTTG
GAGCATCAAA 1560

AAAAAAAAAAAA AAAAAAAAAAAAAA AAAAAAAA

1587

(2) INFORMATION FOR SEQ ID NO:12:

(i) **SEQUENCE CHARACTERISTICS:**

- (A) LENGTH: 472 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Arabidopsis thaliana*

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Branched chain oxoacid dehydrogenase complex
Elalpha

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ala Ile Trp Phe Ala Arg Ser Lys Thr Leu Val Ser Ser Leu Arg His
1 5 10 15

Asn Leu Asn Leu Ser Thr Ile Leu Ile Lys Arg Asp Tyr Ser His Arg
20 25 30

Pro Ile Phe Tyr Thr Thr Ser Gln Leu Ser Ser Thr Ala Tyr Leu Ser
35 40 45

Pro Phe Gly Ser Leu Arg His Glu Ser Thr Ala Val Glu Thr Gln Ala
50 55 60

Asp His Leu Val Gln Gln Ile Asp Glu Val Asp Ala Gln Glu Leu Asp
65 70 75 80

Phe Pro Gly Gly Lys Val Gly Tyr Thr Ser Glu Met Lys Phe Ile Pro
85 90 95

Glu Ser Ser Ser Arg Arg Ile Pro Cys Tyr Arg Val Leu Asp Glu Asp
100 105 110

Gly Arg Ile Ile Pro Asp Ser Asp Phe Ile Pro Val Ser Glu Lys Leu
115 120 125

Ala Val Arg Met Tyr Glu Gln Met Ala Thr Leu Gln Val Met Asp His
130 135 140

Ile Phe Tyr Glu Ala Gln Arg Gln Gly Arg Ile Ser Phe Tyr Leu Thr
145 150 155 160

Ser Val Gly Glu Glu Ala Ile Asn Ile Ala Ser Ala Ala Ala Leu Ser
165 170 175

Pro Asp Asp Val Val Leu Pro Gln Tyr Arg Glu Pro Gly Val Leu Leu
 180 185 190
 Trp Arg Gly Phe Thr Leu Glu Glu Phe Ala Asn Gln Cys Phe Gly Asn
 195 200 205
 Lys Ala Asp Tyr Gly Lys Gly Arg Gln Met Pro Ile His Tyr Gly Ser
 210 215 220
 Asn Arg Leu Asn Tyr Phe Thr Ile Ser Ser Pro Ile Ala Thr Gln Leu
 225 230 235 240
 Pro Gln Ala Ala Gly Val Gly Tyr Ser Leu Lys Met Asp Lys Lys Asn
 245 250 255
 Ala Cys Thr Val Thr Phe Ile Gly Asp Gly Gly Thr Ser Glu Gly Asp
 260 265 270
 Phe His Ala Gly Leu Asn Phe Ala Ala Val Met Glu Ala Pro Val Val
 275 280 285
 Phe Ile Cys Arg Asn Asn Gly Trp Ala Ile Ser Thr His Ile Ser Glu
 290 295 300
 Gln Phe Arg Ser Asp Gly Ile Val Val Lys Gly Gln Ala Tyr Gly Ile
 305 310 315 320
 Pro Lys His Pro Val Trp Asp Gly Thr Asp Ala Leu Ala Val Tyr Ser
 325 330 335
 Ala Val Arg Ser Ala Arg Glu Met Ala Val Thr Glu Gln Arg Pro Val
 340 345 350
 Leu Ile Glu Met Met Thr Tyr Arg Val Gly His His Ser Thr Ser Asp
 355 360 365
 Asp Ser Thr Lys Tyr Arg Ala Ala Asp Glu Ile Gln Tyr Trp Lys Met
 370 375 380
 Ser Arg Asn Pro Val Asn Arg Phe Arg Lys Trp Val Glu Asp Asn Gly
 385 390 395 400
 Trp Trp Ser Glu Glu Asp Glu Ser Lys Leu Arg Ser Asn Ala Arg Lys
 405 410 415
 Gln Leu Leu Gln Ala Ile Gln Ala Ala Glu Lys Trp Glu Lys Gln Pro
 420 425 430
 Leu Thr Glu Leu Phe Asn Asp Val Tyr Asp Val Lys Pro Lys Asn Leu
 435 440 445
 Glu Glu Gln Glu Leu Gly Leu Lys Glu Leu Val Lys Lys Gln Pro Gln
 450 455 460
 Asp Tyr Pro Pro Gly Phe His Val
 465 470

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1319 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Arabidopsis thaliana*

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Branched chain oxoacid dehydrogenase complex
Elbeta

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTCTTCACCC ACCAAAAGTA GCAAACCTTT GCCACCTAAA AATCTTACCA
GTTGGGTGAA 60

AGTTGCCAAA ATAGAGCTTG CTTTGTGCGC AATCCTATAT TTTTCAGATT
GATTGTTGGT 120

GGGTTTGTGT AAATGGCGGC TCTTTTAGGC AGATCTGCC GGAAACTGAG
TTTCCGAGC 180

TTGACTCAGC GAGCTAGGAG GGTATCGACG GAAACTGGAA AACCATTGAA
TCTATACTCT 240

GCTATTAATC AAGCGCTTCA CATCGCTTTG GACACCGATC CTCGGTCTTA
TGTCCTTGGG 300

GAAGACGTTG GCTTTGGTGG AGTCTTTCGC TGTACAACGT GTTTAGCTGA
ACGATTCCGG 360

AAAAACCGTG TCTTCAATAC TCCTCTTTGT GAGCAGGGCA TTGTTGGATT
TGGCATTGGT 420

CTAGCAGCAA TGGGTAATCG AGCAATTGTA GAGATTCACT TTGCAGATTA
TATATATCCT 480

GCTTTGATC AGATTGTTAA TGAAGCTGCA AAGTTCAGAT ACCGAACTGG
TAACCAATTC 540

AACTGTGGAG GACTTACGAT AAGAGCACCA TATGGAGCAG TTGGTCATGG
TGGACATTAC 600

CATTACAAT CCCCTGAAGC TTTCTTTTGC CATGTCCTGT GTATTAAGGT
TGTTATCCCT 660

CGGAGTCCAC GAGAAGCAAA GGGACTGTTG TTGTCATGTA TCCGTGATCC
AAATCCCGTT 720

GTTTCTCTCG AACCAAAGTG GCTGTATCGT CAAGCAGTAG AAGAAGTCCC

TGAGCATGAC 780

TATATGATAC CTTTATCAGA AGCAGAGGTT ATAAGAGAAG GCAATGACAT
TACACTGGTT 840GGATGGGGAG CTCAGCTTAC CGTTATGGAA CAAGCTTGTC TGGACGCGGA
AAAGGAAGGA 900ATATCATGTG AACTGATAGA TCTCAAGACA CTGCTTCCTT GGGACAAAGA
AACCGTTGAG 960GCTTCAGTTA AAAAGACTGG CAGACTTCTT ATAAGCCATG AAGTCCTGT
AACAGGAGGT 1020TTTGAGCAG AGATCTCTGC AACAACTCTG GAACGTTGCT TTTTGAAGTT
AGAAAGCTCA 1080GTAAGCAGAG TTTGTGGTCT GGATACTCCA TTTCCTCTTG TGTTTGAACC
ATTCTACATG 1140CCCACCAAGA ACAAGATATT GGATGCAATC AAATCGACTG TGAATTACTA
GCCGTACTAT 1200CTGTAGTTTA CTGTTTACAC TAGGACTAAT GTAATCGCAT GTCTTTGTGA
TCAATTCGTC 1260TAATGTAACA CTACCGATTA ACTTTAATGA ATTCAAGAT AACGAAAAAA
AAAAAAA 1319

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 352 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Arabidopsis thaliana*

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Branched chain oxoacid dehydrogenase complex
 Elbeta

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Ala Ala Leu Leu Gly Arg Ser Cys Arg Lys Leu Ser Phe Pro Ser
 1 5 10 15

Leu Thr His Gly Ala Arg Arg Val Ser Thr Glu Thr Gly Lys Pro Leu
 20 25 30

Asn Leu Tyr Ser Ala Ile Asn Gln Ala Leu His Ile Ala Leu Asp Thr
 35 40 45

Asp Pro Arg Ser Tyr Val Phe Gly Glu Asp Val Gly Phe Gly Gly Val
 50 55 60
 Phe Arg Cys Thr Thr Gly Leu Ala Glu Arg Phe Gly Lys Asn Arg Val
 65 70 75 80
 Phe Asn Thr Pro Leu Cys Glu Gln Gly Ile Val Gly Phe Gly Ile Gly
 85 90 95
 Leu Ala Ala Met Gly Asn Arg Ala Ile Val Glu Ile Gln Phe Ala Asp
 100 105 110
 Tyr Ile Tyr Pro Ala Phe Asp Gln Ile Val Asn Glu Ala Ala Lys Phe
 115 120 125
 Arg Tyr Arg Ser Gly Asn Gln Phe Asn Cys Gly Gly Leu Thr Ile Arg
 130 135 140
 Ala Pro Tyr Gly Ala Val Gly His Gly Gly His Tyr His Ser Gln Ser
 145 150 155 160
 Pro Glu Ala Phe Phe Cys His Val Pro Gly Ile Lys Val Val Ile Pro
 165 170 175
 Arg Ser Pro Arg Glu Ala Lys Gly Leu Leu Ser Cys Ile Arg Asp
 180 185 190
 Pro Asn Pro Val Val Phe Phe Glu Pro Lys Trp Leu Tyr Arg Gln Ala
 195 200 205
 Val Glu Glu Val Pro Glu His Asp Tyr Met Ile Pro Leu Ser Glu Ala
 210 215 220
 Glu Val Ile Arg Glu Gly Asn Asp Ile Thr Leu Val Gly Trp Gly Ala
 225 230 235 240
 Gln Leu Thr Val Met Glu Gln Ala Cys Leu Asp Ala Glu Lys Glu Gly
 245 250 255
 Ile Ser Cys Glu Leu Ile Asp Leu Lys Thr Leu Leu Pro Trp Asp Lys
 260 265 270
 Glu Thr Val Glu Ala Ser Val Lys Lys Thr Gly Arg Leu Leu Ile Ser
 275 280 285
 His Glu Ala Pro Val Thr Gly Gly Phe Gly Ala Glu Ile Ser Ala Thr
 290 295 300
 Ile Leu Glu Arg Cys Phe Leu Lys Leu Glu Ala Pro Val Ser Arg Val
 305 310 315 320
 Cys Gly Leu Asp Thr Pro Phe Pro Leu Val Phe Glu Pro Phe Tyr Met
 325 330 335
 Pro Thr Lys Asn Lys Ile Leu Asp Ala Ile Lys Ser Thr Val Asn Tyr
 340 345 350

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1450 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Arabidopsis thaliana*

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Branched chain oxoacid dehydrogenase complex E2

(ix) FEATURE:

- (A) NAME/KEY: mRNA
- (B) LOCATION: 33..1450
- (D) OTHER INFORMATION: /function = "Open reading frame"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGAAACAAAC ACACGGACCA ACCGTTTCATA ACAATGATCG CGCGACGGAT
CTGGCGAAGC 60

CACCGGTTTC TCCGCCCATC CAGCTCGTCA TCTGTTTGCT CTCGCGCGTT
CCGGGTACCG 120

GAGTATCTTT CTCAGTCGTC TTCTCTCCG GCGTCGCGCC CATTCTTTGT
TCACCTCCC 180

ACTTTGATGA AATGGGGTGG AGGAAGTAGA AGCTGGTTTT CGAACGAAGC
CATGGCCACT 240

GATTCAAATT CAGGGTTAAT TGATGTGCCA CTAGCTCAAA CTGGGGAAGG
TATTGCTGAA 300

TGTGAGCTTC TCAAGTGGTT TGTCAAAGAG GGAGATTCTG TGGAAGAGTT
TCAGCCACTC 360

TGTGAAGTTC AGAGCGATAA AGCAACTATA GAGATCACA GTGCTTTTAA
AGGGAAAGTG 420

GCTCTGATTT CACATTCTCC AGGTGACATT ATTAAGGTTG GAGAGACTCT
GGTTAGGTTG 480

GCGGTTGAAG ACTCGCAGGA TTCGCTTCTA ACCACTGATA GTTCAGAAAT
TGTAACCTCG 540

GGAGGTTCAA AGCAGGGAAC AGAAAATCTT CTGAGAGCTC TCTCAACGCC
TGCGGTTCGT 600

AACCTTGCAA AAGACCTTGG CATAGATATC AATGTTATAA CTGGAACCTG
TAAAGATGGT 660

AGAGTTTGA AAGAGGATGT TCTCCGGTTT AGTGACCAGA AAGGATTGT
AACAGATTCA 720

GTTTCTTCTG AGCATGCTGT TATAGGAGGA GACTCGGTTT CCACTAAAGC
TAGTAGTAAC 780

TTTGAAGATA AAACAGTTCC TCTAAGGGA TTCAGCCGAG CAATGGTCAA
GACAATGACT 840

ATGGCTACAA GTGTACCGCA TTTTCATTTT GTTGAAGAGA TAAACTGCCA
CTCATTGTG 900

GAGCTCAAGC AGTTCTTCAA AGAGAACAAT ACAGATTCCA CCATCAACAA
CACTTTTCTT 960

CCTACTTTAA TCAAGTCTCT GTCAATGGCT CTAACCAAAT ATCCCTTCGT
GAATAGTTGC 1020

TTCAACGCGG AATCTCTCGA GATCATTCTC AAAGGTTAC ATAATATTGG
AGTTGCAATG 1080

GCCACTGAAC ATGGCCTTGT CGTTCCTAAT ATAAAGAATG TTCAGTCATT
ATCTCTGCTA 1140

GAGATAACCA AAGAGCTGTG CCGGTTACAA CATTTGGCAG CAAACAACAA
ACTTAACCCC 1200

GAGGATGTGA CTGGTGGAAC CATAACTCTG AGTAACATTG GAGCAATTGG
TGGTAAATTC 1260

GGATCCCTTC TTTTAACTT ACCGGAAGTT GCAATCATCG TTCTTGGAAG
AATCGAGAAA 1320

GTTCCAAAAT TCTCAAAAGA AGGAACTGTC TATCCTGCAT CGATAATGAT
GGTTAACATT 1380

GCTGCGGATC ATAGAGTTCT AGATGGGGCA ACGGTAGCTC GGTTTTGCTG
CCAGTGGAAA 1440

GAGTATGTCG 1450

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 483 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Arabidopsis thaliana

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Branched chain oxoacid dehydrogenase complex

E2

(ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION: 1..61

(D) OTHER INFORMATION: /note = "Mitochondrial targeting peptide"

(ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION: 5..13

(D) OTHER INFORMATION: /note = "Peroxisomal targeting peptide"

(ix) FEATURE:

(A) NAME/KEY: Domain

(B) LOCATION: 79..124

(D) OTHER INFORMATION: /note = "Lipoyl domain"

(ix) FEATURE:

(A) NAME/KEY: Binding-site

(B) LOCATION: 183..217

(D) OTHER INFORMATION: /note = "Subunit binding domain"

(ix) FEATURE:

(A) NAME/KEY: Domain

(B) LOCATION: 262..483

(D) OTHER INFORMATION: /note = "Inner catalytic domain"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met Ile Ala Arg Arg Ile Trp Arg Ser His Arg Phe Leu Arg Pro Phe
 1 5 10 15

Ser Ser Ser Ser Val Cys Ser Pro Pro Phe Arg Val Pro Glu Tyr Leu
 20 25 30

Ser Gln Ser Ser Ser Ser Pro Ala Ser Arg Pro Phe Val His Pro
 35 40 45

Pro Thr Leu Met Lys Trp Gly Gly Gly Ser Arg Ser Trp Phe Ser Asn
 50 55 60

Glu Ala Met Ala Thr Asp Ser Asn Ser Gly Leu Ile Asp Val Pro Leu
 65 70 75 80

Ala Gln Thr Gly Glu Gly Ile Ala Glu Cys Glu Leu Lys Trp Phe
 85 90 95

Val Lys Glu Gly Asp Ser Val Glu Glu Phe Gln Pro Leu Cys Glu Val
 100 105 110

Gln Ser Asp Lys Ala Thr Ile Glu Ile Thr Ser Arg Phe Lys Gly Lys
 115 120 125

Val Ala Leu Ile Ser His Ser Pro Gly Asp Ile Ile Lys Val Gly Glu
 130 135 140

Thr Leu Val Arg Leu Ala Val Glu Asp Ser Gln Asp Ser Leu Leu Thr

145 150 155 160
 Thr Asp Ser Ser Glu Ile Val Thr Leu Gly Gly Ser Lys Gln Gly Thr
 165 170 175
 Glu Asn Leu Leu Gly Ala Leu Ser Thr Pro Ala Val Arg Asn Leu Ala
 180 185 190
 Lys Asp Leu Gly Ile Asp Ile Asn Val Ile Thr Gly Thr Gly Lys Asp
 195 200 205
 Gly Arg Val Leu Lys Glu Asp Val Leu Arg Phe Ser Asp Gln Lys Gly
 210 215 220
 Phe Val Thr Asp Ser Val Ser Ser Glu His Ala Val Ile Gly Gly Asp
 225 230 235 240
 Ser Val Ser Thr Lys Ala Ser Ser Asn Phe Glu Asp Lys Thr Val Pro
 245 250 255
 Leu Arg Gly Phe Ser Arg Ala Met Val Lys Thr Met Thr Met Ala Thr
 260 265 270
 Ser Val Pro His Phe His Phe Val Glu Glu Ile Asn Cys Asp Ser Leu
 275 280 285
 Val Glu Leu Lys Gln Phe Phe Lys Glu Asn Asn Thr Asp Ser Thr Ile
 290 295 300
 Lys His Thr Phe Leu Pro Thr Leu Ile Lys Ser Leu Ser Met Ala Leu
 305 310 315 320
 Thr Lys Tyr Pro Phe Val Asn Ser Cys Phe Asn Ala Glu Ser Leu Glu
 325 330 335
 Ile Ile Leu Lys Gly Ser His Asn Ile Gly Val Ala Met Ala Thr Glu
 340 345 350
 His Gly Leu Val Val Pro Asn Ile Lys Asn Val Gln Ser Leu Ser Leu
 355 360 365
 Leu Glu Ile Thr Lys Glu Leu Ser Arg Leu Gln His Leu Ala Ala Asn
 370 375 380
 Asn Lys Leu Asn Pro Glu Asp Val Thr Gly Gly Thr Ile Thr Leu Ser
 385 390 395 400
 Asn Ile Gly Ala Ile Gly Gly Lys Phe Gly Ser Leu Leu Asn Leu
 405 410 415
 Pro Glu Val Ala Ile Ile Val Leu Gly Arg Ile Glu Lys Val Pro Lys
 420 425 430
 Phe Ser Lys Glu Gly Thr Val Tyr Pro Ala Ser Ile Met Met Val Asn
 435 440 445
 Ile Ala Ala Asp His Arg Val Leu Asp Gly Ala Thr Val Ala Arg Phe
 450 455 460

Cys Cys Gln Trp Lys Glu Tyr Val Glu Lys Pro Glu Leu Leu Met Leu
465 470 475 480

Gln Met Arg

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: PDC E1alpha chloroplast targeting peptide forward primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGGCCCCATA TGGCGACGGC TTTC

24

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: PDC E1alpha chloroplast targeting peptide reverse primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGGGCGGCCG CTAATAACCA CCTAAC

26

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: PDC Elalpha chloroplast targeting reverse primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGGCCCGCGG CCGCTGATCA TTTGGTTCAG CAG

33

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: BCOADC Elalpha forward primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGGCCCGCGG CCGCTGATCA TTTGGTTCAG CAG

33

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: BOCADC Elalpha reverse primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGGCCCGTCG ACTCAAACAT GAAAGCCAGG

30

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: PDC E1beta chl targ pept + E2 bind reg linker
forward primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GGGCCCCATA TGTCTTCGAT AATC

24

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: PDC E1beta chl targ pept + E2 bind reg linker
reverse primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGGCCCCTCG AGACCTTCTT GAAGAGC

27

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: BCOADC E1beta forward primer w/o E2 binding
site

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GGGCCCACCG GTTTTGGCAT TGGTCTA

27

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
(B) CLONE: BCOADC E1beta reverse primer w/o E2 binding site

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GGGCCCGAAT TCTCATTACT AGTAATTCAC AGT

33

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
(B) CLONE: PDC E2 chloroplast targeting peptide forward

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GGGCCCCATA TGGCGGTTTC TTCT

24

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
(B) CLONE: PDC E2 chloroplast targeting peptide reverse

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GGGCCCCCAT GGCAATTCA GGATTCCT

28

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
(B) CLONE: PDC E1beta targeting peptide + E2 binding
region forward

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GGGCCCCATA TGTCTTCGAT AATC

24

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
(B) CLONE: PDC E1alpha chloroplast targeting peptide
forward primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GGGCCCCCAT GCGACGGCT TTCGCT

26

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
(B) CLONE: PDC E1alpha chloroplast targeting peptide
reverse primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GGGCCCTGAT CATATTATTG GTGGATTGCT T

31

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: BCOADC E1beta forward primer with E2 binding site

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGGCCCCTCG AGATCGCTTT GGACACC

27

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Plastid E1alpha targeting peptide reverse primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GGGCCCCGCG CCGCATTATT GGTGGATTGC TT

32

What Is Claimed Is:

1. An isolated DNA molecule, comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence shown in SEQ ID NO:1, or the complement thereof;

(b) a nucleotide sequence that hybridizes to said nucleotide sequence of (a) under a wash stringency equivalent to 0.5X SSC to 2X SSC, 0.1% SDS, at 55-65°C, and which encodes a polypeptide having enzymatic activity similar to that of *Arabidopsis thaliana* plastid pyruvate dehydrogenase complex E1 α subunit;

(c) a nucleotide sequence encoding the same genetic information as said nucleotide sequence of (a), but which is degenerate in accordance with the degeneracy of the genetic code; and

(d) a nucleotide sequence encoding the same genetic information as said nucleotide sequence of (b), but which is degenerate in accordance with the degeneracy of the genetic code.

2. A recombinant vector, comprising said isolated DNA molecule of claim 1.

3. A host cell transformed with said recombinant vector of claim 2.

4. An isolated polypeptide having the amino acid sequence of SEQ ID NO.:2.

5. An isolated DNA molecule, comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence shown in SEQ ID NO:3, or the complement thereof;

(b) a nucleotide sequence that hybridizes to said nucleotide sequence of (a) under a wash stringency equivalent to 0.5X SSC to 2X SSC, 0.1% SDS, at 55-65°C, and which encodes a polypeptide having enzymatic activity similar to that of *Arabidopsis thaliana* plastid pyruvate dehydrogenase complex E1 β subunit;

(c) a nucleotide sequence encoding the same genetic information as said nucleotide sequence of (a), but which is degenerate in accordance with the degeneracy of the genetic code; and

(d) a nucleotide sequence encoding the same genetic information as said nucleotide sequence of (b), but which is degenerate in accordance with the degeneracy of the genetic code.

6. A recombinant vector, comprising said isolated DNA molecule of claim 5.

7. A host cell transformed with said recombinant vector of claim 6.

8. An isolated polypeptide having the amino acid sequence of SEQ ID NO.:4.

9. An isolated DNA molecule, comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence shown in SEQ ID NO:5, or the complement thereof;

(b) a nucleotide sequence that hybridizes to said nucleotide sequence of (a) under a wash stringency equivalent to 0.5X SSC to 2X SSC, 0.1% SDS, at 55-65°C, and which encodes a polypeptide having enzymatic activity similar to that of *Arabidopsis thaliana* plastid pyruvate dehydrogenase complex E2 component;

(c) a nucleotide sequence encoding the same genetic information as said nucleotide sequence of (a), but which is degenerate in accordance with the degeneracy of the genetic code; and

(d) a nucleotide sequence encoding the same genetic information as said nucleotide sequence of (b), but which is degenerate in accordance with the degeneracy of the genetic code.

10. A recombinant vector, comprising said isolated DNA molecule of claim 9.

11. A host cell transformed with said recombinant vector of claim 10.

12. An isolated polypeptide having the amino acid sequence of SEQ ID NO.:6.

13. An isolated DNA molecule, comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence shown in SEQ ID NO:11, or the complement thereof;

(b) a nucleotide sequence that hybridizes to said nucleotide sequence of (a) under a wash stringency equivalent to 0.5X SSC to 2X SSC, 0.1% SDS, at 55-65°C, and which encodes a polypeptide having enzymatic activity similar to that of *Arabidopsis thaliana* branched chain 2-oxoacid dehydrogenase complex E1 α subunit;

(c) a nucleotide sequence encoding the same genetic information as said nucleotide sequence of (a), but which is degenerate in accordance with the degeneracy of the genetic code; and

(d) a nucleotide sequence encoding the same genetic information as said nucleotide sequence of (b), but which is degenerate in accordance with the degeneracy of the genetic code.

14. A recombinant vector, comprising said isolated DNA molecule of claim 13.

15. A host cell transformed with said recombinant vector of claim 14.

16. An isolated polypeptide having the amino acid sequence of SEQ ID NO.:12.

17. An isolated DNA molecule, comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence shown in SEQ ID NO:13, or the complement thereof;

(b) a nucleotide sequence that hybridizes to said nucleotide sequence of (a) under a wash stringency equivalent to 0.5X SSC to 2X SSC, 0.1% SDS, at 55-65°C, and which encodes a polypeptide having enzymatic activity similar to that of *Arabidopsis thaliana* branched chain 2-oxoacid dehydrogenase complex E1 β subunit;

(c) a nucleotide sequence encoding the same genetic information as said nucleotide sequence of (a), but which is degenerate in accordance with the degeneracy of the genetic code; and

(d) a nucleotide sequence encoding the same genetic information as said nucleotide sequence of (b), but which is degenerate in accordance with the degeneracy of the genetic code.

18. A recombinant vector, comprising said isolated DNA molecule of claim 17.

19. A host cell transformed with said recombinant vector of claim 18.

20. An isolated polypeptide having the amino acid sequence of SEQ ID NO.:14.

21. The isolated DNA molecule of claim 17, wherein the naturally occurring branched chain oxoacid dehydrogenase complex E2 component binding region thereof is replaced with the E2 component binding region of a plastid pyruvate dehydrogenase complex E1 β subunit.

22. The isolated DNA molecule of claim 21, wherein said plastid pyruvate dehydrogenase complex E1 β subunit has the sequence shown in SEQ ID NO.:3.

23. A recombinant vector, comprising said isolated DNA molecule of claim 22.

24. A host cell transformed with said recombinant vector of claim 23.

25. An isolated DNA molecule, comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence shown in SEQ ID NO:15, or the complement thereof;

(b) a nucleotide sequence that hybridizes to said nucleotide sequence of (a) under a wash stringency equivalent to 0.5X SSC to 2X SSC, 0.1% SDS, at 55-65°C, and which encodes a polypeptide having enzymatic activity similar to that of *Arabidopsis thaliana* branched chain 2-oxoacid dehydrogenase complex E2 component;

(c) a nucleotide sequence encoding the same genetic information as said nucleotide sequence of (a), but which is degenerate in accordance with the degeneracy of the genetic code; and

(d) a nucleotide sequence encoding the same genetic information as said nucleotide sequence of (b), but which is degenerate in accordance with the degeneracy of the genetic code.

26. A recombinant vector, comprising said isolated DNA molecule of claim 25.

27. A host cell transformed with said recombinant vector of claim 26.

28. An isolated polypeptide having the amino acid sequence of SEQ ID NO.:16.

29. A plant, a plastid of which comprises the following polypeptides:

- an enzyme that enhances the biosynthesis of 2-oxobutyrate;
- a branched chain oxoacid dehydrogenase complex E1 α subunit;
- a branched chain oxoacid dehydrogenase complex E1 β subunit; and
- a branched chain oxoacid dehydrogenase complex E2 component.

30. The plant of claim 29, wherein said branched chain oxoacid dehydrogenase complex E1 α subunit has the sequence shown in SEQ ID NO.:12, said branched chain oxoacid dehydrogenase complex E1 β subunit has the sequence shown in SEQ ID NO.:14, or said branched chain oxoacid dehydrogenase complex E2 component has the sequence shown in SEQ ID NO.:16.

31. The plant of claim 29, wherein said plastid further comprises the following polypeptides:

- a β -ketothiolase;
- a β -ketoacyl-CoA reductase; and
- a polyhydroxyalkanoate synthase.

32. The plant of claim 31, the genome of which comprises introduced DNAs encoding said polypeptides, wherein each of said introduced DNAs is operatively linked to a targeting peptide coding region capable of directing transport of said polypeptide encoded thereby into a plastid.

33. A method of producing P(3HB-co-3HV) copolymer, comprising growing said plant of claim 32, and recovering P(3HB-co-3HV) copolymer produced thereby.

34. A plant, a plastid of which comprises the following polypeptides:

- an enzyme that enhances the biosynthesis of 2-oxobutyrate;
- a branched chain oxoacid dehydrogenase complex E1 α subunit;
- a branched chain oxoacid dehydrogenase complex E1 β subunit;
- a branched chain oxoacid dehydrogenase complex E2 component; and
- a dihydrolipoamide dehydrogenase E3 component.

35. The plant of claim 34, wherein said branched chain oxoacid dehydrogenase complex E1 α subunit has the sequence shown in SEQ ID NO.:12, said branched chain oxoacid dehydrogenase complex E1 β subunit has the sequence shown in SEQ ID NO.:14, or said branched chain oxoacid dehydrogenase complex E2 component has the sequence shown in SEQ ID NO.:16.

36. The plant of claim 34, wherein said plastid further comprises the following polypeptides:

- a β -ketothiolase;
- a β -ketoacyl-CoA reductase; and
- a polyhydroxyalkanoate synthase.

37. The plant of claim 36, the genome of which comprises introduced DNAs encoding said polypeptides, wherein each of said introduced DNAs is operatively linked to a targeting peptide coding region capable of directing transport of said polypeptide encoded thereby into a plastid.

38. A method of producing P(3HB-co-3HV) copolymer, comprising growing said plant of claim 37 and recovering P(3HB-co-3HV) copolymer produced thereby.

39. A plant, a plastid of which comprises the following polypeptides:

- an enzyme that enhances the biosynthesis of 2-oxobutyrate;
- a branched chain oxoacid dehydrogenase complex E1 α subunit; and
- a branched chain oxoacid dehydrogenase complex E1 β subunit, the naturally occurring E2 binding region of which is replaced with the E2 binding region of a plastid pyruvate dehydrogenase complex E1 β subunit.

40. The plant of claim 39, wherein said branched chain oxoacid dehydrogenase complex E1 α subunit has the sequence shown in SEQ ID NO.:12.

41. The plant of claim 39, wherein said plastid further comprises the following polypeptides:

- a β -ketothiolase;
- a β -ketoacyl-CoA reductase; and
- a polyhydroxyalkanoate synthase.

42. The plant of claim 41, the genome of which comprises introduced DNAs encoding said polypeptides, wherein each of said introduced DNAs is operatively

linked to a targeting peptide coding region capable of directing transport of said polypeptide encoded thereby into a plastid.

43. A method of producing P(3HB-co-3HV) copolymer, comprising growing said plant of claim 42 and recovering P(3HB-co-3HV) copolymer produced thereby.

APPENDIX A

CATCTCTTGT	TCTCTCCGCC	CATCTCTGCT	CTCTTTTATT	TTCCACAGAA	GTTTTTTTT	60
TTTTTTCOGA	ATTCCGTTAA	TCTCATTGGG	GTTTCCATTG	ATAGCAATGG	CGACGGCTTT	120
CGCTCCCACT	AGCTCACTG	CCACGGTTCC	TCTGCATGGA	TCCCATGAGA	ATCGTCTCTT	180
GCTTCCGATC	CGATTGGCTC	CTCCTTCTTC	TTTCTCCGGA	TCCACCCGTT	CCTCTCTCCT	240
TGCGACACTC	ARTCACTCCA	ACGCCACCCG	TGATCTCCCT	GTCGTCCTCT	TCCAGGAAGT	300
TGTCAAGGAG	ARGCAATCCA	CCAATAATAC	CAGCCTGTTG	ATAACCAAG	AGGAAGGATT	360
GGAGTTGTAT	GAGATATATG	TACTAGGTAG	ATCTTTTCGAA	GACATGTGTG	CTCAAAATGT	420
TTACCGAGGC	AGATCTTTTG	GTTTGTGTCA	CTTGTACAA	GGCCAAGAGG	CTGTTTCTAC	480
TGGCTTTATC	AGATCTCTTA	CCAAGTCTGA	CTCTGTCTGT	AGTACCTACC	GTGACCATGT	540
CCATGCCCTC	AGCAAGGTG	TCTCTGCTCG	TGCTGTTATG	AGCGAGCTCT	TGGCAAGGT	600
TACTGGATGC	TGCAGAGGCC	AAGGTGGATC	CATGCACATG	TTCTCCAAAG	AACACAACAT	660
GCTTGGTGGC	TTTGCTTTTA	TTGGTGAAGG	CATTCTCTGC	GCCACTGGTG	CTGCCCTTTAG	720
CTCCAAAGIAC	AGGAGGGAAG	TCTTGAACAA	GGATTGTGAT	GATGTCACGTG	TGCGCTTTTT	780
CGGAGATGGA	ACTTGTAAAC	ACGGACAGTT	CTTCGAGTGT	CTCAACATGG	CTGCTCTCTA	840
TAAACTGCCT	ATTATCTTTG	TTGTCGAGAA	TAACTTGTGG	GCCATTGGGA	TGCTCACTT	900
GAGAGCCACT	CTGACCCCG	AGATTGGAA	GAAAGGTCTC	GCATTGGGA	TGCCTGGTGT	960
TCATGTTGAC	GATATGGATG	TCTTGAAGGT	CAGGGAAGTC	GCTAAGGAAG	CTGTCACTAG	1020
AGCTAGAGAG	GGAGAAGGTC	CAACCTTGGT	TGAATGTGAG	ACTTATAGAT	TCAGAGGACA	1080
CTCCTTGGCT	GATCCCGATG	AGCTCCGTGA	TGCTGCTGAG	AAAGCCAAAT	ACGGCGGTAG	1140
AGACCCCAATC	CAAGCATTTA	AGAAGTATTT	GATAGAGAAC	AAGCTTGCAA	AGGAAGCAGA	1200
GCTAAGTCCA	ATAGAGAAAA	AGATAGACGA	GTTGGTGGAG	GAAGCGGTTG	AGTTTGCAGA	1260
CGCTAGTCCA	CAGCCCGGTC	GCAGTCAGTT	GCTAGAGAAT	GTGTTTGCTG	ATCCAAAAGG	1320
ATTGGAATTT	GAACCTGATG	GACGGTACAG	ATGTAGGAGC	CCCAAGTTTA	CCGAGGACAC	1380
AGCTCAAGTC	TGAGAGACA	AGTTTAACCA	TAAGCTGTCT	ACTGTCTCTT	CGATGTTTCT	1440
ATATATCTTA	TTAGTTAAA	TGCTACAGAG	AATCAGTTTG	AATCATTTCG	ACTTTTTGCT	1500
TAAAAAANA	AAAAAANA	AAAAAANA				1530

APPENDIX B

<u>MATAFAPTKL TATVPLHGSH ENRLLLPIRL APPSSFLGST RSLSLRRLNH SNATRRSPVV</u>	60
<u>SVQEVVKEKQ STNNTSLLIT KEEGLELYED MILGRSFEDM CAQMYYRGKM FGFVHLYNGQ</u>	120
EAVSTGFIKL LTKSDSVST YRDHVHALSK GVSARAVMSE LFGKVTGCCR GQGGSMHMF	180
KEHNMLGGFA FIGEGIPVAT GAAFSSKYRR EVLKQDCDDV TVAFFGDGTC NNGQFFECLN	240
MAALYKLPII FVVENNLWAI GMSHLRATSD PEIWKKGPAF GMPGVHVDGM DVLKVREVA	300
EAVTRARRGE GPTLVECETY RFRGHSADP DELRDAAEKA KYAARDPIAA LKKYLIENKL	360
AKAEELKSIE KKIDELVEEA VEFADASPQF GRSQLENVF ADPKGFGIGP DGRYRCEDPK	420
FTEGTAQV	428

APPENDIX C

GAARAAATGT	CTTCGATAAT	CCATGGAGCT	GGAGCTGCTA	CGACGAGGTT	ATCGACGTTT	60
AATTCGGTGG	ATTCCAAGAA	ACTCTTCGTT	GCTCCTTCTC	GCACAAATCT	TTCACTGAGG	120
AGCCAGAGAT	ATATAGTGGC	TGGATCTGAT	GCGAGTAAGA	AGAGCTTTGG	TTCTGGACTT	180
AGAGTTCTGC	ACTCTCAGAA	ATTGATTCCA	AATGCTGTTG	CGACGAAGGA	GSCGGATACG	240
TCTGCGAGCA	CTGGACATGA	ACTATTGCTT	TTGAGGAGCTC	TTCAGGAAGG	TCTGGAAGAA	300
GAGATGGACA	GAGATCCACA	TGTATGTGTT	ATGGGTGAAG	ATGTTGCCCA	TTACGAGAGT	360
TCCTACAAGG	TAACCAAAGG	CCTTGCTGAT	AAATTTGGTG	ACCTCAGGGT	TCTCGACACT	420
CCTATTTTGT	AAATGTCATT	CACCGGTATG	GGCATTGGAG	CTGCCATGAC	TGCTTAAGA	480
CCCCTTATTG	AAGGTATGAA	CATGGGTTTC	CTCCTCCTCG	CCTTCAACCA	AATCTCCAAC	540
AACTGTGGAA	TGCTTCACTA	CACATCCGGT	GGTCAGTTTA	CGATCCCGGT	TGTATCCGTT	600
GGAGCTGTGG	GAGTGGGACG	CCAGCTTGGT	GCTGAGCATT	CACAGAGGTT	AGAATCTTAC	660
TTTCAGTCCA	TCCTGGGATG	CCAGATGGTT	GCTTGCTCAA	CTCCTTACAA	CGCCAAAGGG	720
TTGATGAAG	CCGCAATAAG	AAGCGAGAAC	CCTGTGATTC	TGTTCAACA	CGTGCTGCTT	780
TACATCTCA	AGGAGAAAT	CCCGGATGAA	GATTACATCT	GTAACCTTGA	AGAAGCTGAG	840
ATGGTCAGAC	CTGGCGAGCA	CATTACCATC	CTCACTTACT	CGCGAATGAG	GTACCATGTG	900
ATGCAGGCAG	CAAAAACCTCT	GGTGAACAAA	GGGTATGACC	CCGAGGTTAT	CGACATCAGG	960
TCACCTGAAC	CGTTCGACCT	TCACACAATT	GGAAACTCGG	TGAAGAAAAC	ACATCGGGTT	1020
TTGATCGTGG	AGGAGTGTAT	GAGAACCCTG	GGGATTGGGG	CAAGTCTTAC	AGCTGCCATC	1080
AACGAGAACC	TTATGACTA	CTTAGATGCT	CCGGTGATGT	GTTTATCTTC	TCAAGACGTT	1140
CCTACACCTT	ACGCTGGTAC	ACTGGAGGAG	TGGACCGTGG	TTCACCCGGC	TCAGATCGTG	1200
ACCGCTGTGC	AGCAGCTTTG	CCAGTAAATT	CATATTTATC	CGATGAACCA	TATATTCA	1260
TTTACTCTTC	CATTTCCTTT	CTCTGAGCT	TAGTTCTTAA	AGATTTGCT	TAAGATGGTT	1320
TGTTTTTGT	AAAGTTTGT	TCCTTTGTTG	TGCTTTTAA	TATGTTTGT	AACTCAGAAAT	1380
TTTGTTTGT	TATTTTATC	TCCCACTTTC	TTTTAAAAA	AAAAAATAA	AAAAAATAA	1440
A						1441

APPENDIX D

<u>MSSIIHGAGA ATTTLSTFNS VDSKKLFVAP SRTNLSVRSQ RYIVAGSDAS KKSFGSGLRV</u>	60
<u>RHSQKLIPNA VATKEADTSA STGHELLLFE ALQEGLEEEM DRDPHVCVMG EDVGHYGGSY</u>	120
KVTKGLADKF GDLRVLDTP I CENAF TGMGI GAAMTGLRPV IEGMNMGFL LAFNQISNNC	180
GMLHYTSGGQ FTIPVVIRGP GGVGRQLGAE HSQRLESYFQ SIPGIQMVAC STPYNAKGLM	240
KAAIRSENPV ILFEHVLLYN LKEKIPDEDY ICNLEEAEMV RPGEHITILT YSRMRYHVMQ	300
AAKTLVNKG YDPEVIDIRSL KPFDLHTIGN SVKKTHRVL I VEECMRTGGI GASLTAINE	360
NFHDYLDAPVMCLSSQDVPT PYAGTLEEWTVVQPAQIVTA VEQLCQ	406

APPENDIX E

GCGCGATCTG GTTTCCTAGA TCCAAAACCC TTGTTTCTAG CTTCGACCAT 50
 AATCAAAATT TGTGACCAAT TCTCATAAAA CGTCAATTAAT CTCATCGTCC 100
 CATCTCTAT ACACATCTCT AGTATCTCTC AACGGCGTAT TTGAGTCCTT 150
 TCGGTACCTT CGGTCTGAG TCTACGGGCG TCGACACACA GGTCTGATCT 200
 TTGGTTCAG AGTCTGATGA AGTCTGATGC CAGGACTCG ATTTCGACAG 250
 AGGCAAGTC GGTTCACCAT CGGACATGA ATTCTATCCG GAATCATCTT 300
 CAGGAGCTT TCTCTGTAC CGGCTCTCTG ACGAGACCG ACGATCATC 350
 CCGATACCG ATTCTATCTC GGTCTGATGA AACTCTGCTG TTGATGATGA 400
 CGACCAATG GCGCGCTAC AGTCAATGA TCACTCTCTC TAGCAGCTC 450
 AACCTCAGG ACGATATCTT TTTTATCTTA CTCTCGTCCG AGACGAGCC 500
 ATTACATCG CTTCACGAG TGTCTCTAGT CCTGACGAG TGTCTTACG 550
 TCGATACGA GACCTGAGG TTCTTTTGTG GGTGCTCTC AGGTGAGG 600
 AGTCTGCTA TCACTGTCTT GCGACCAAG CTGATATCG CAAAGCGAG 650
 CAAATGCTA TTCAATTCGG TTCTCAATCT CTATATCTT TCACTATCT 700
 CTCTCCATTT GCGCGCAGG TTCTCAAGG TGTCTGATTT GGTATCTCT 750
 TGAAATGGA CAGCAGCAT GCTCTGATG TTCACTCAT CCGCATCTG 800
 GCGACAGCG AGGACATTT TCAAGCGGA TTCAATTTTG CCGCGTAT 850
 GCGAGCTCG GTTGTCTTA TTCTGCTGA CAGCGTGG GGTATGAT 900
 CTCATCTCT ACACAGTCT ACAGCTGAT CATATGAT GAAAGTCA 950
 GGTATATAT GTCTACCT CAGCTCTGA AATGCTCTA ACAGACAA 1000
 GAGCTCTCT CATCTGATG ATCACTATA GATGAGCA TCATCTTCA 1050
 TCACTGATT CACTAGTA CAGGCGCGG GATCAATCC AGTCTCTGA 1100
 AATGCTGGA AACCTGTGA ATAGTCTTG GAATGCTG GAGATACG 1150
 GATGCTGGG TCGGAGAT GATCTCAGG TAGCTCTTA CCGACGAAA 1200
 CAGCTCTCT AACGATCTA GGTCTGAGG AAGTGGGA AACACCAT 1250
 GACAGCTG TTAAAGCTG TATATGATG TAAAGCAG AACCTCAG 1300
 ACGAGACT TTGTTGAGG GATATGAT ACGACAGC TCAGATAT 1350
 CCTCTGCTT TTCTGTTG AATCTAGAG AACTGTGTT TTAATATCC 1400
 TCGCGACCG GCAATGAT ATCAAGCTC TCATCTCA CTATTTAT 1450
 TGTCCAGTA TCGATAGTA ATCAAGTAT AATGTAGA CCAGCATTTG 1500
 GAGCATCAA AAAAAAAAAA AAAAAAAAAA AAAAAA 1587

APPENDIX F

AIWFARSKTL VSSLRHNLNL STILIKRDYS HRPIFYTTSQ LSSTAYLSPF	50
GSLRHSTAV ETOADHLVQQ IDEVDAQELD FPGGKVGYS EMKFIPSSS	100
RRIPCVRVLD EDGRIIPDSO FIPVSEKLAV RMYEQMATLQ VMDHIFYEAO	150
ROGRISFYLT SVGEEAINIA SAAALSPDDV VLPQYREPGV LLWRGFTLEE	200
TPP binding site	
FANOCFGNKA DYKGROMPT HYGSNRLNYF TISSPIATQL PQAAGVGYSL	250
BCOADC E1β binding site	
KMDKKNACTV TFIGDGGTSE GDFHAGLNFA AVMEAPVVF CRNNGWAIST	300
HISEQFRSDG IVVKGQAYGI PKHPVWDGTD ALAVYSAVRS AREMAVTEOR	350
PVLIEMMTYR VGHSTSDDS TKYRAADEIO YWKMSRNPVN RFRKWVEDNG	400
WWSEEDESKL RSNARKOLLO AIOAAEKWEK OPLTELFNDV YDVKPNLEE	450
OELGLKELVK KOPQDYPPGF HV	472

APPENDIX G

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
TTCTTCACCC	ACCAAAAGTA	GCAAAOCTTT	GCCACCTAAA	AATCTTACCA	50
GTITGGGIGAA	AGTITGCCAA	ATAGAGCTTG	CTTTTGTCGC	AATCCTATAT	100
TTTTTCAGATT	GATTGTGTGT	GGGTTTGIGT	AAATGGGGGC	TCFTTTAGGC	150
AGATCCTGCC	GGAACCTGAG	TTTTTOGAGC	TTGACTCACG	GAGCTAGGAG	200
GGTATCGAOG	GAAACTGGAA	AACCAITGAA	TCTATACTCT	GCTATTATCT	250
AAGCGCTTCA	CATCGCTTTG	GACACCGATC	CTGGGTCCTA	TGCTTTGGG	300
GAAGAOGITG	GCTTTGGTGG	AGTCTTTTCG	TGTACAACTG	GTTTAGCTGA	350
AOGATTOGGG	AAAAACOGTG	TCITTCATAC	TCCTCTTTGT	GAGCAGGGCA	400
TTGTITGGATT	TGGCATGTGT	CTAGCAGCAA	TGGTAAATCG	AGCAATTGTA	450
GAGATTTCAGT	TTGCAGATT	TATATATCCT	GCTTTTGAAT	AGATTGTTPA	500
TGAAGCTGCA	AAGTTCAGAT	AOCGAAGTGG	TACCAATTC	AACCTGTGGAG	550
GACTTACGAT	AACAGCAACA	TATGGAGCAG	TTGTCATGG	TGGACATTAC	600
CATTTCACAT	COOCTGAAGC	TTTCTTTTTC	CATGTCCCTG	GTATTAGGTT	650
TGTTATCCCT	CGGATGCCAC	GAGAAGCAAA	GGGACTGTTC	TTGTTCATGTA	700
TOOGTATCC	AAATCCOGIT	GTITTCITTCG	AAOCAAGTIG	GCTGTATCGT	750
CAAGCAGTAG	AAGAAGTCC	TGAGCATGAC	TATATGATAC	CTTATTCAGA	800
AGCAGAGGTT	ATAGAGAGAG	GCAATGACAT	TACACTGGTT	GGATGGGGAG	850
CTCAGCTTAC	CGTATGGA	CAAGCTTGTC	TGGAOCCGA	AAAGGAAGGA	900
ATATCATGTG	AACTGATAGA	TCTCAAGACA	CTGCTTCCTT	GGGCAAAAGA	950
AACCGTGTAT	GCTTCAGTTA	AAAAGACTGG	CAGACTTCCT	ATTAAGCCATG	1000
AAGCTCCTGT	AACAGGAGGT	TTTGGAGCAG	AGATCTCTGC	AACAAITCTG	1050
GAAOGITGCT	TTTTTGAAGTT	AGAAGCTCCA	GTAAGCAGAG	TTTGTGGTCT	1100
GGATACTCCA	TTTCTCTCTG	TGTTTGAACC	ATTCTACATG	CCCAOCAAAG	1150
ACAAGATATT	GGATGCAATC	AAATOGACTG	TGAATTACTA	GCCGTACTAT	1200
CTGTAGTTTA	CTGTTTACAC	TAGGACTAAT	GTAATGCGAT	GCTCTTGTGA	1250
TCAATTOGTC	TAAATGTAACA	CTACCGATT	ACTTTAATGA	ATTTCAGAT	1300
AACGAAAAAA	AAAAAAA				1319

APPENDIX H

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
MAALLGRSCR	KLSFFSLTHG	ARRVSTETGK	FINLYSAINQ	ALHIALDIDP	50
RSYVFGEDVG	FGGVFRCTTG	LAERFGKNRV	FNIPLCBQGI	VGRGIGLAAM	100
GNRAIVEIQF	ADYIYPADFQ	IVNEAAKFRY	RSQNQFNCGI	LITRAPYGAU	150
GGGHYHSQS	PEAFFCHVPG	IKWVLRSPR	EAKGLLLSCI	RDNFVVFFE	200
EKWLRYQAVE	EVPEHDYMLP	LSAEVIREG	NDITLVGWA	QLIVMEQACL	250
DAEKEGLSCE	LIDLKILLPW	DKEIVEASVK	KIGRLLISHE	APVTGGFGAE	300
ISATILERC	LKLEAPVSRV	QGLDTPFPLV	PEPFYMPTRN	KILDAIKSTV	350
NY					352

FIG. 1

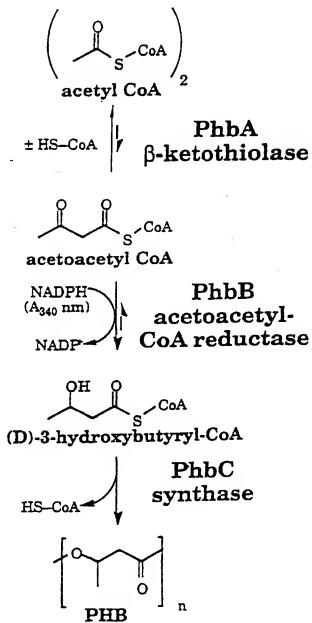
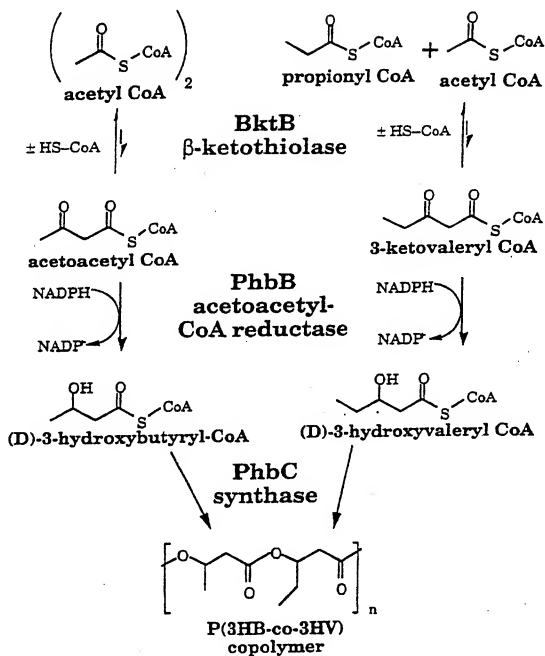


FIG. 2



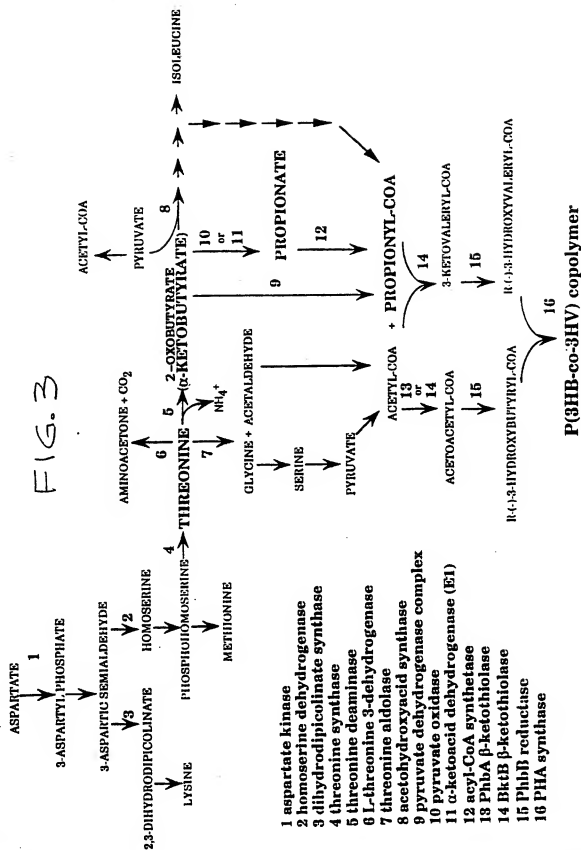


FIG. 4

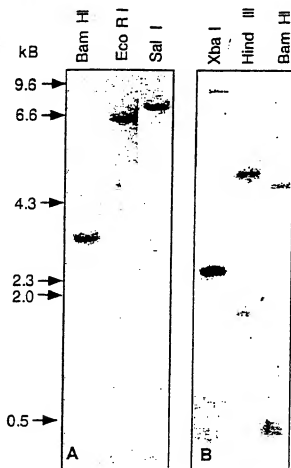


FIG. 5

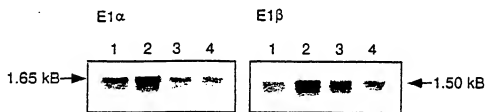


FIG. 6A

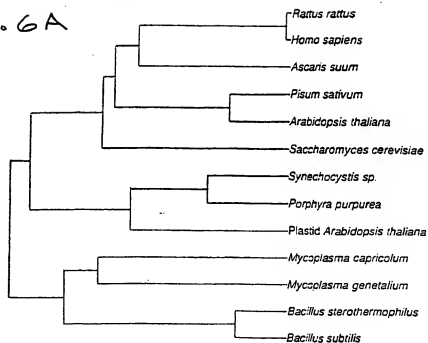


FIG. 6B

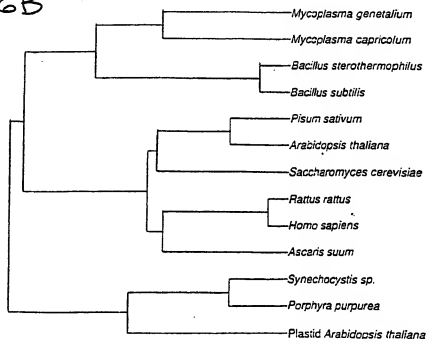
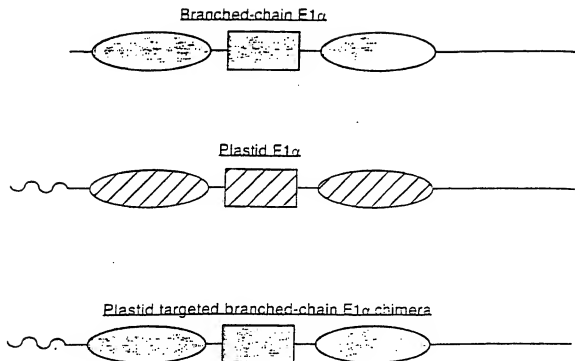
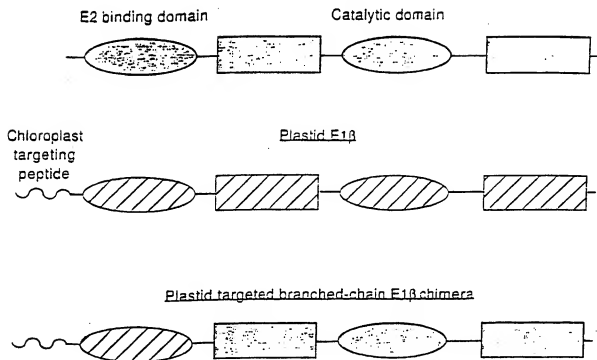


FIG. 7A



Construct 1: Attach the chloroplast targeting peptide of E1 α to the mature portion of the branched-chain E1 α . This creates a plastid targeted branched-chain E1 α chimera.

FIG. 7B

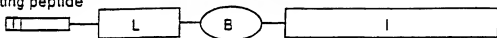
Branched-chain E1 β 

Construct 2: Replace the N-terminus of the branched-chain E1 β (including the E2 binding domain) with the N-terminus of the plastid E1 β (including the chloroplast targeting peptide and the plastid E2 binding domain). This creates a plastid targeted branched-chain E1 β chimera.

FIG. 7C

Peroxisomal/mitochondrial
targeting peptide

Branched-chain E2



Chloroplast
targeting peptide

Plastid E2

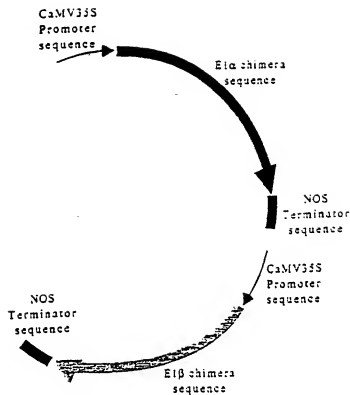


Plastid targeted branched-chain E2



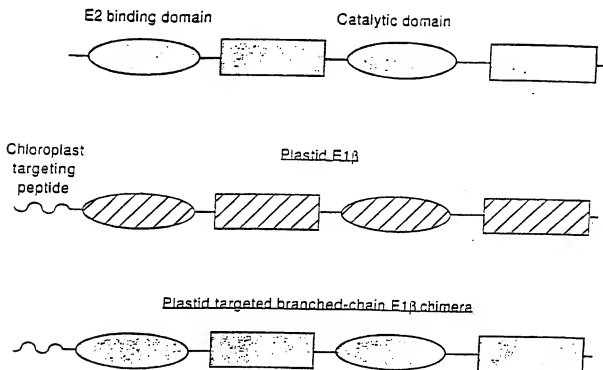
Construct 3: Attach the chloroplast targeting peptide of the plastid E2 to the mature portion of the branched-chain E2, to create a plastid targeted branched-chain E2 chimera.

FIG. 7D



Construct 4. Mega plasmid coding for both chimeric (plastid targeted branched-chain) subunits of the PDH. Attach the E1 α chimeric sequence to the E1 β chimeric sequence with transcription terminator and promoter sequences between the two.

FIG. 7E

Branched-chain E1 β 

Construct 5: Attach the chloroplast targeting peptide of the plastid E1 β to the mature portion of the branched-chain E1 β . This creates a plastid targeted branched-chain E1 β chimera.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/13406

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/53 C12N15/54 C12N15/82 C12N9/02 C12N9/10 C12N5/10 C12P7/62 A01H5/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C12P A01H		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEWMAN T. ET AL.: "EST AC N65566" EMBL DATABASE, 8 March 1996, XP002086082 Heidelberg see the whole document ---	1-3
X	NEWMAN T. ET AL.: "EST AC R29966" EMBL DATABASE, 11 August 1995, XP002086083 Heidelberg see the whole document ---	5-7
X	NEWMAN T. ET AL.: "EST AC W43179" EMBL DATABASE, 27 June 1996, XP002086084 Heidelberg see the whole document --- -/--	9-11
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "Z" document member of the same patent family		
Date of the actual completion of the international search 27 November 1998		Date of making of the international search report 14/12/1998
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel: (+31-70) 340-2040; Tx: 31 651 epo nl; Fax: (+31-70) 340-3016		Authorized officer Kania, T

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 98/13406

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO 95 05472 A (UNIV MICHIGAN) 23 February 1995 see the whole document ---	29-43
A	WO 93 02187 A (UNIV MICHIGAN) 4 February 1993 see the whole document ---	29-43
A	WO 92 19747 A (ICI PLC) 12 November 1992 see the whole document ---	29-43
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P,X	<p>JOHNSTON M L ET AL: "Cloning and molecular analyses of the Arabidopsis thaliana plastid pyruvate dehydrogenase subunits." PLANT BIOLOGY '97: 1997 ANNUAL MEETINGS OF THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS AND THE CANADIAN SOCIETY OF PLANT PHYSIOLOGISTS, JAPANESE SOCIETY OF PLANT PHYSIOLOGISTS AND THE AUSTRALIAN SOCIETY OF PLANT PHYSIOLOGISTS, VANCOUVER, BRITISH COLU, XP002086091 see abstract</p>	1-8
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Category *	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

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